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Guinea-Pig Milk-Protein Synthesis

ISOLATION AND CHARACTERIZATION OF MESSENGER RIBONUCLEIC ACIDS FROM LACTATING MAMMARY GLAND AND IDENTIFICATION OF CASEINS AND PRE-α-LACTALBUMIN AS TRANSLATION PRODUCTS IN HETEROLOGOUS CELL-FREE SYSTEMS

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1. The major milk proteins synthesized by the lactating mammary gland of the guinea pig were identified and designated as caseins A, B and C and α-lactalbumin, with estimated mol.wts. of 28000, 25500, 20500 and 14500 respectively. 2. Antisera to the total casein fraction and to α-lactalbumin were prepared from rabbits. The milk proteins were also iodinated with either ^{131}I or ^{125}I. 3. A poly(A)-rich RNA fraction was isolated from lactating guinea-pig mammary glands. Isolation was by affinity chromatography on oligo(dT)–cellulose. 4. Examination of this RNA fraction by electrophoresis on polyacrylamide gels containing formamide indicated three major species 1, 2 and 3, with estimated mol.wts. of 5.4×10^5, 4.5×10^5 and 3.3×10^5, and the apparent absence of rRNA species. 5. The poly(A)-rich RNA stimulated protein synthesis in heterologous cell-free systems based on wheat germ, Krebs II ascites-tumour cells, and the latter supplemented with an initiation factor-3 fraction from rabbit reticulocyte ribosomes. 6. Between 80 and 90% of the protein synthesis directed by the mRNA was for milk proteins. 7. Analysis of the proteins immunoprecipitated by the α-lactalbumin antiserum showed in the wheat-germ system that the product was a protein with a molecular weight greater than that of α-lactalbumin, whereas in the ascites-tumour-cell systems both this protein and α-lactalbumin were found. When the larger protein was treated with CNBr and the resulting peptides were examined, it was shown that the extra peptide was at the *N*-terminus. This and other evidence is adduced for the initial translation product of α-lactalbumin being a precursor with an addition of about ten amino acids at the *N*-terminus. 8. Similar analysis of the casein immunospecific proteins produced under the direction of mRNA indicated that the products had a molecular weight that was apparently a little smaller than that of the caseins synthesized *in vivo*. This was not consistent with higher-molecular weight casein precursors. 9. Possible explanations for the results obtained are discussed, especially in terms of the physiological significance of the pre-α-lactalbumin as a secretory protein.

To investigate and resolve the subtleties of gene expression in eukaryotes, a system is required in which certain well-defined proteins are produced in response to specific stimuli. Isolation of mRNA molecules for such proteins, and the subsequent synthesis *in vitro* of DNA complementary to these mRNA molecules, provide biochemical probes, which may then be used to investigate the complexities of gene expression at the molecular level. This approach has been used by several workers to investigate systems such as the effect of oestrogen on gene expression in the chick oviduct (O'Malley & Means, 1974; Cox *et al.*, 1974; McKnight *et al.*, 1975), and the mechanisms regulating globin gene expression in a variety of tissues and cell lines (Ross *et al.*, 1974; Harrison *et al.*, 1974).

We have chosen the mammary gland as a model system in which to investigate both the control of protein synthesis at the levels of transcription and translation, and also the subsequent mechanisms and subcellular sites of post-translational modification of secretory proteins.

Total mRNA fractions directing the synthesis of a variety of milk proteins have been isolated from the lactating mammary gland of the ewe (Gaye & Houdebine, 1975) and rat (Rosen *et al.*, 1975*a*), and

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earlier work from our laboratory (Campbell *et al.*, 1973) demonstrated the presence of mRNA for α-lactalbumin in polyribosomal RNA isolated from lactating guinea-pig mammary gland. We now report not only the purification of a total mRNA fraction from lactating guinea-pig mammary gland, which directs the synthesis of all the major milk-protein fractions in a variety of heterologous systems, but also the synthesis *in vitro* of α-lactalbumin as a precursor molecule modified at the *N*-terminus.

Experimental

Preparation of solutions

All Tris-containing buffers are expressed in molar concentrations of Tris and were adjusted to the required pH at the stated temperature with 4M-HCl. All solutions were prepared in double-distilled water. Both phenol (under N_2) and *NNN'N'*-tetramethylethylenediamine (*in vacuo*) were redistilled immediately before use. Acrylamide and *NN'*-methylenebisacrylamide were recrystallized in chloroform and acetone respectively as described by Loening (1967).

As a safeguard against degradation of RNA species by the ribonucleases the following precautions were taken. (1) Gloves were worn for all experiments involving RNA isolation, and subsequent experiments with mRNA fractions. (2) All buffers used during RNA-isolation procedures were autoclaved in the presence of 0.025% diethyl pyrocarbonate at 104kPa (15lb/in²) for 20min. (3) As a routine glassware was sterilized overnight at 180°C. (4) Plastic pipette tips and centrifuge tubes were sterilized at 69kPa (10lb/in²) for 20min.

Scintillation fluid contained 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene.

Materials

Tuck no. 1 mice and Dunkin Hartley guinea pigs were from A. Tuck and Son, Battlesbridge, Essex, U.K., and New Zealand White rabbits were from L. Moore, Thornton, Bradford, Yorks., U.K.; guinea-pig milk was from Porcellus Animal Breeding, Heathfield, Sussex, U.K. Sephadex G-100, G-75, G-25 and poly(U)–Sepharose were from Pharmacia (G.B.), Ealing, London W5 5SS, U.K.; Whatman DEAE-cellulose DE-52 and Whatman glass-fibre (GF/C) filter discs were from Reeve Angel Scientific, London SE1 6BD, U.K.; oligo(dT)–cellulose T-2 was from Collaborative Research Inc., Waltham, MA, U.S.A.; Freund's complete and incomplete adjuvant were from Difco laboratories, Detroit, MI, U.S.A.; formamide (Fluka) was from Fluorochem, Glossop, Derbyshire, U.K.; Stainsall was from Eastman Kodak Co., Rochester, NY, U.S.A.; heparin (Pularin) was from Evans Medical, Speke, Liverpool, U.K.; Nembutal was from Abbott Laboratories, Queenborough, Kent, U.K.; NCS solubilizer was from Hopkin and Williams, Romford RM1 1HA, Essex, U.K.; liquid-scintillation fluors were from Fisons Scientific Apparatus, Loughborough, Leics., U.K.; lactate dehydrogenase was from Boehringer (London), Lewes, E. Sussex, U.K.; bovine serum albumin (fraction V) and goat IgG* to rabbit IgG was from Miles Laboratories, Stoke Poges, Slough SL2 4LY, U.K.; ovalbumin, trypsin, creatine kinase, spermidine, L amino acids, dithiothreitol, ATP, GTP and Coomassie Brilliant Blue R were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; L-[3,5-^{3}H]-tyrosine (53Ci/mmol), ^{3}H-labelled amino acid mixture, and carrier-free ^{131}I and ^{125}I were from The Radiochemical Centre, Amersham, Bucks., U.K.; all other chemicals and solvents were from BDH Chemicals, Poole, Dorset, U.K. All reagent chemicals were A.R. grade.

Freshly milled (untoasted) wheat germ was a gift from Mr. Bellow, Healthilife (Bradford), Bradford, U.K.; Krebs II ascites cells were a gift from Dr. T. S. Work, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

Methods

Preparation of a post-nuclear RNA fraction from lactating guinea-pig mammary gland. Glands from lactating guinea pigs 3–6 days *post partum*, killed by cervical dislocation, were dissected into ice-cold breakage buffer, consisting of 50mM-Tris/HCl, pH7.4 at 4°C, containing 0.25M-sucrose, 25mM-NaCl and 5mM-magnesium acetate. The glands were rinsed in fresh buffer, blotted, passed through a tissue mincer, and then disrupted in 2.5vol. of breakage buffer by four strokes of a Teflon pestle (0.05mm clearance) in a glass homogenizer tube operating at 1000rev./min. The homogenate was then centrifuged at $4000g_{av.}$ at 4°C for 10min, the resulting upper fatty layer discarded, and the remaining supernatant retained as a source of post-nuclear RNA. NaCl and EDTA were then added to the supernatant to final concentrations of 100mM and 10mM respectively, and the supernatant was finally adjusted to 1% (w/v) sodium dodecyl sulphate by dropwise addition of a 10% solution with constant stirring at 20°C. After 10min at 20°C the solution was extracted with an equal volume of phenol/chloroform (1:1, v/v) saturated with 10mM-sodium acetate, pH6.0, containing 100mM-NaCl and 1mM-EDTA essentially as described by Perry *et al.* (1972). The aqueous and phenolic phases were separated by centrifugation at $18000g_{av.}$ at 10°C for 10min, the upper aqueous

* Abbreviations: IgG, immunoglobulin G; RNAase, ribonuclease.

phase was retained, and the interphase and phenolic phase were re-extracted with an equal volume of breakage buffer containing 1% (w/v) sodium dodecyl sulphate and adjusted to 100mM and 10mM with respect to NaCl and EDTA respectively.

The pooled aqueous phases were extracted a further four times with phenol/chloroform/sodium acetate; the final aqueous phase was adjusted to 2% (w/v) potassium acetate, pH5.0, and the RNA was allowed to precipitate overnight at −20°C after the addition of 2.5vol. of ethanol. The resulting precipitate was collected by centrifugation at $18000g_{av.}$ at −10°C for 10min, washed three times by successive resuspension and sedimentation in 100–200vol. of 70% (v/v) ethanol containing 40mM-NaCl, dried *in vacuo*, and dissolved in water at a final concentration of 60–100 E_{260} units/ml.

Protein was determined by the method of Warburg & Christian (1942).

Isolation of mRNA. Poly(A)-containing RNA was selectively bound to oligo(dT)–cellulose in a similar manner to that described by Nakazato & Edmonds (1974). The post-nuclear RNA fraction dissolved in water was diluted with an equal volume of 20mM-Tris/HCl, pH7.4 at 20°C, containing 200mM-NaCl, 20mM-EDTA and 0.4% sodium dodecyl sulphate. To this was added oligo(dT)–cellulose [2000 E_{260} units of RNA/g of oligo(dT)–cellulose] and the slurry gently stirred for 20–30min at 20°C. The oligo(dT)–cellulose was sedimented by centrifugation at $3000g_{av.}$ for 5min at 20°C, the supernatant decanted, and the oligo(dT)–cellulose resuspended in binding buffer, consisting of 10mM-Tris/HCl, pH7.4 at 20°C, containing 100mM-NaCl, 10mM-EDTA and 0.2% sodium dodecyl sulphate. The resulting slurry was poured into a water-jacketed column (28cm×1.2cm) and eluted with binding buffer until no E_{260} could be detected in the eluate. The poly(A)-containing RNA fraction was recovered by elution at 60°C with 10mM-Tris/HCl, pH7.4 at 20°C, containing 10mM-EDTA and 0.2% sodium dodecyl sulphate. To remove minor rRNA contaminants, the eluate was immediately adjusted to 100mM-NaCl and the binding, washing and elution cycle repeated with a fresh portion of oligo(dT)–cellulose. The final eluate was adjusted to 0.2M-NaCl, and the poly(A)-containing RNA allowed to precipitate overnight at −20°C after the addition of 2vol. of ethanol. The resulting precipitate of RNA was collected by sedimentation and washed three times in 70% ethanol containing 40mM-NaCl as described above. The final sedimented precipitate was dried *in vacuo*, and dissolved in sterile water (not treated with diethyl pyrocarbonate) and stored in 100μl batches at −70°C.

Guinea-pig liver tRNA. tRNA was prepared by the method of Rogg *et al.* (1969), by using livers snap-frozen in liquid N_2 and stored at −70°C until required. Purified tRNA was dissolved in sterile water at 120 E_{260} units/ml and stored in batches at −70°C.

Isolation of globin mRNA from rabbit reticulocyte polyribosomes. Salt-washed polyribosomes (see under 'Preparation of a source of reticulocyte initiation factor-3') were resuspended in 10mM-sodium acetate buffer, pH6.0, containing 100mM-NaCl and 1mM-EDTA, at a concentration of 20 E_{260} units/ml, and the solution was adjusted to 0.5% sodium dodecyl sulphate by dropwise addition of a 10% solution. The solution was then extracted repeatedly with phenol/chloroform/sodium acetate as described for the mammary-gland preparation, and the resulting RNA precipitated with ethanol, washed three times with 70% ethanol containing 40mM-NaCl, and dried *in vacuo* before mRNA isolation by affinity chromatography on oligo(dT)–cellulose as described by Aviv & Leder (1972). The final ethanol-precipitated mRNA fraction was dissolved in sterile water and stored at −70°C without apparent loss of activity over a period of 18months. Globin mRNA prepared in this manner contained minor 28S and 18S rRNA contaminants.

Molecular-weight determinations of mammary-gland mRNA species by polyacrylamide-gel electrophoresis in the presence of formamide. Electrophoresis was as described by Haines *et al.* (1974) with minor modifications. Formamide was extracted with an equal volume of diethyl ether, and the residual ether removed by the passage overnight of a steady stream of N_2 through the formamide. The formamide was then deionized by gentle stirring with Amberlite-MB3 (6g/100ml) for 2h at room temperaure. Acrylamide (880mg), *NN'*-methylenebisacrylamide (150mg), diethylbarbituric acid (92mg) and *NNN'N'*-tetramethylethylenediamine (0.06ml) were dissolved in formamide (20ml) and the pH was adjusted to 9.0 with 1M-HCl. Then 0.2ml of 18% (w/v) ammonium persulphate was added and the volume made up to 25ml with formamide. The 9cm gels were immediately poured into 15cm×0.4cm gel tubes and overlaid with water. After 30min the water overlay was replaced with a 5cm column of buffered formamide (deionized formamide containing 20mM-barbiturate/HCl, pH9.0) and the gels were left overnight at room temperature. Freeze-dried RNA samples were dissolved in 20μl of buffered formamide containing a trace of Bromophenol Blue and 10% (w/v) sucrose. Samples were loaded with a Pasteur pipette through the buffered formamide. Electrophoresis was performed at room temperature for 90min at 2.5mA/gel with continuous buffer circulation, with 20mM-NaCl as the reservoir buffer. Gels were stained overnight in 0.005% Stainsall dissolved in 50% (v/v) formamide, destained in water and scanned at 570nm by using a Unicam SP.1800 recording spectrophotometer with gel-scanning optics. Molecular-weight determinations were based on the comparative mobilities of the following RNA species, with respect to Bromophenol

Blue: 28S and 18S rRNA [mol.wt. 1.9×10^6 and 0.71×10^6 respectively (McConkey & Hopkins, 1969)]; 10S and 9S mRNA [mol.wt. 0.227×10^6 and 0.202×10^6 respectively (Gould & Hamlyn, 1973)]; 5S rRNA [mol.wt. 0.41×10^5 (Forget & Weissman, 1967)].

Preparation of heterologous cell-free systems. (i) Wheat germ. Freshly milled wheat germ was processed as described by Roberts & Paterson (1973) and the S-30 (30000*g* supernatant) fraction stored in 250μl batches in liquid N_2. (ii) Krebs II ascites. The preparation of a Krebs II ascites cell-free system was a modification of that of Mathews & Korner (1970). Krebs II ascites cells, maintained by routine passage in male Schofield mice, were harvested into 10vol. of ice-cold wash buffer consisting of 35mM-Tris/HCl, pH7.4 at 4°C, containing 146mM-NaCl, and washed free of contaminating erythrocytes by repeated resuspension and sedimentation at $200g_{av.}$ for 5min at 4°C. The final cell suspension was centrifuged at $1000g_{av.}$ for 10min, and the sedimented cells were resuspended in an equal volume of 10mM-Tris/HCl, pH7.5 at 4°C, containing 1.5mM-magnesium acetate, 10mM-KCl, 1mM-dithiothreitol, and allowed to swell for 5min at 4°C. The cells were then disrupted in a glass homogenizer tube by using 20–25 strokes of a Teflon pestle (0.01mm clearance) at 1000rev./min. Breakage of cells was ascertained by phase-contrast microscopy. Then 0.1vol. of 300mM-Mops [3-(*N*-morpholino)propanesulphonic acid], pH8.0 at 20°C, containing 1.25M-KCl, 50mM-magnesium acetate and 10mM-dithiothreitol was added, and the homogenate was centrifuged at $30000g_{av.}$ for 10min at 4°C. The resulting upper lipid layer was discarded and the remaining supernatant (S-30) adjusted to 1mM-ATP, 0.2mM-GTP, 1.63mg of creatine phosphate/ml and 0.2mg of creatine kinase/ml, and incubated at 37°C for 30min. The preincubated S-30 fraction was then clarified by filtration through a small glass-wool plug, and 15–20ml loaded on to a column (2.6cm×34cm) of Sephadex G-25 (medium grade), pre-equilibrated in 30mM-Mops, pH7.4, at 4°C containing 125mM-KCl, 5mM-magnesium acetate, 1mM-dithiothreitol. The column was eluted at 60ml/h, and the most turbid void-volume fractions were pooled and stored as 300μl batches in liquid N_2, with no loss of activity for periods of up to 18 months.

Preparation of a source of reticulocyte initiation factor-3. A high reticulocyte count was induced in male New Zealand white rabbits (2.5–3.0kg body wt.) by five subcutaneous injections (each 0.25ml/kg body wt.) of 2.5% (w/v) phenylhydrazine containing 1mM-glutathione at 24h intervals. The animals were then rested for 48h, anaesthetized with Nembutal containing heparin (6000 units/kg body wt.) and the cells harvested by cardiac puncture. Cells and plasma were separated by centrifugation at $2000g_{av.}$ for 10min at 4°C, the plasma was discarded, and the cells were washed three times by resuspension and sedimentation in 4vol. of 10mM-Tris/HCl, pH7.5 at 4°C, containing 154mM-NaCl. After the second wash, the upper layer of leucocytes was removed by using a Pasteur pipette. After the final wash the erythrocytes were lysed in an equal volume of water for 5min at 4°C. Cell debris was removed by centrifugation at $12000g_{av.}$ for 10min at 4°C and polyribosomal material harvested from the supernatant by centrifugation at $165000g_{av.}$ for 3h at 4°C in the Ti50 rotor of a Beckman L2-65B ultracentrifuge. The sedimented polyribosomes were resuspended at $150E_{260}$ units/ml in factor buffer consisting of 5mM-Tris/HCl, pH7.5 at 4°C, containing 0.5mM-EDTA, 1mM-dithiothreitol and 0.25M-sucrose and adjusted to 0.5M-KCl by the dropwise addition of 3.0M-KCl. The suspension was stirred gently at 4°C for 45min, and the salt-washed polyribosomes were removed by centrifugation in an MSE Super Speed 65 ultracentrifuge at $305000g_{av.}$ for 90min at 4°C in the 10×10ml fixed-angle titanium rotor. The sedimented polyribosomes were used as a source of total rRNA and globin mRNA, and the supernatant was adjusted by the addition of factor buffer to 0.35M-KCl. The supernatant was then passed through a DEAE-cellulose column (6cm×5.5cm), equilibrated in factor buffer containing 0.35M-KCl, and the unbound fraction was retained and dialysed overnight at 4°C against three changes (100vol./change) of factor buffer containing 0.1M-KCl. The dialysed crude initiation-factor preparation, containing 250mg of protein, was loaded at 20ml/h on to a column (2.0cm×15.0cm) of DEAE-cellulose equilibrated in factor buffer containing 0.1M-KCl, and the column then washed exhaustively with the same buffer. The unbound fraction was discarded, and the bound fraction eluted by raising the salt concentration to 0.25M-KCl and retained as a source of reticulocyte initiation factor-3. This was concentrated by the addition of solid $(NH_4)_2SO_4$ added with gentle stirring to 80% saturation at 4°C (Dawson *et al.*, 1969). After 30min, the precipitated proteins were collected by centrifugation at $18000g_{av.}$ for 20min at 4°C, and the sedimented material was dissolved in a minimal volume of factor buffer containing 0.1M-KCl. This was dialysed overnight at 4°C against three changes (100vol./change) of the same buffer, and finally stored in 50μl batches in liquid N_2 at a protein concentration of 7mg/ml.

Preparation of milk-protein fractions from guinea-pig milk. A total casein fraction was prepared by acid precipitation of guinea-pig milk as described for ewe's milk by Soulier *et al.* (1975). The resulting fraction was either stored as a dry powder at −20°C, or solubilized by careful addition of 0.1M-NaOH to pH7.0 with constant vigorous stirring at room temperature, and stored at −20°C.

α-Lactalbumin was prepared by acid precipitation

and Sephadex G-100 chromatography as described by Fairhurst *et al.* (1971).

Antibody techniques. Antibodies were raised in male New Zealand White rabbits, by intramuscular injection at multiple sites. The first injection (1.5ml total injected per animal) consisted of 1vol. of antigen suspended in 2vol. of Freund's complete adjuvant emulsified as described by Waynforth (1969). Subsequent injections at 2, 4 and 8 weeks were made by using incomplete Freund's adjuvant. Antibodies were raised against total casein and α-lactalbumin. The amount of antigen was increased during the injection sequence (casein: 4, 8, 8 and 12mg; α-lactalbumin 2, 4, 8 and 12mg). At 3–4 weeks after the final injection, animals were bled from the ear vein and serum from clotted samples was tested for precipitating antibody by titration of a fixed volume of serum against various amounts of ^{125}I-labelled antigen.

Control (preimmunization) serum, and antisera to total casein and α-lactalbumin, were tested for cross-reaction, by titration against ^{125}I-labelled casein and ^{125}I-labelled α-lactalbumin. As a routine, 100μl batches of serum were titred against increasing amounts of iodinated antigen contained in 100μl of 0.15M-NaCl. The assay mixture was incubated at 37°C for 30min, and then overnight at 4°C. Any precipitate was sedimented by centrifugation at 3000$g_{av.}$ at 4°C for 15min, the supernatant discarded and the precipitate washed twice by successive resuspensions and sedimentation in 1ml of 0.15M-NaCl. The final precipitate was resuspended in 1ml of 0.15M-NaCl and collected under suction on a glass-fibre disc (Whatman GF/C; 2.5cm diam.). Each disc was washed under suction with one 5ml portion of 0.15M-NaCl, washed briefly with ethanol, dried, placed in glass vials each containing 10ml of toluene-based scintillation fluid and measured for radioactivity in a Beckman model SL 230 liquid-scintillation spectrometer.

When a reasonable titre was obtained, up to 50ml of blood was collected from each animal and the serum stored in batches at −20°C, without apparent loss of activity over a period of 12 months.

Cell-free protein synthesis and product analysis. Three heterologous assay systems were used, one derived from wheat germ, a second from Krebs II ascites cells, and a third using the Krebs II ascites cell-free system, but with the addition of rabbit reticulocyte initiation factor-3.

Cell-free synthesis by wheat-germ extract was done in a final volume of 50μl at 21°C, for 75min. The incubation mixture contained 20mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid], pH7.5, 60mM-KCl, 2.5mM-magnesium acetate, 0.2mM-spermidine, 2mM-dithiothreitol, 2.4mM-2-mercaptoethanol, 1mM-ATP, 0.2mM-GTP, 8mM-creatine phosphate, 2μg of creatine kinase, 1.0μCi of either L-[3,5-^{3}H]tyrosine (specific radioactivity 53Ci/mmol) or a mixture of ^{3}H-labelled amino acids containing 15 labelled amino acids of varying specific radioactivity, 25μM of each of the common L amino acids not present in the labelled form, 0.005E_{260} unit of mRNA and 5–10μl of wheat-germ S-30 fraction.

Cell-free synthesis using a S-30 fraction from Krebs II ascites cells was carried out in a final volume of 100μl at 30°C for 90min. The incubation mixture contained 20mM-Mops, pH7.5, 80mM-KCl, 3mM-magnesium acetate, 1.2mM-dithiothreitol, 1mM-ATP, 0.2mM-GTP, 20μg of creatine kinase, 4.6mM-creatine phosphate, 2.5μCi of L-[3,5-^{3}H]tyrosine (specific radioactivity 53Ci/mmol), 50μM of each of the common L amino acids not present in the labelled form, 0.32E_{260} unit of guinea-pig liver tRNA, 0.025E_{260} unit of mRNA and 10–15μl of ascites cell S-30 fraction.

Cell-free synthesis by Krebs II ascites cells in the presence of reticulocyte initiation factor-3 was carried out as described for ascites cells alone, with adjustments and additions to the incubation mixture, giving final concentrations of: 120mM-KCl, 3.5mM-magnesium acetate, 1.4mM-dithiothreitol, 0.05M-sucrose and 0.1mM-EDTA.

Determination of total mRNA activity and subsequent product analysis was carried out in an identical manner for all heterologous cell-free systems.

To determine total incorporation of ^{3}H-labelled amino acid residues into protein, synthesis was stopped by the addition of 1ml of 10% (w/v) trichloroacetic acid, followed by a 20min incubation at 90°C. Samples were cooled at 4°C for 20min, and the precipitated material was collected under suction on glass-fibre discs (Whatman GF/C; 2.5cm diam.). Each disc was then washed individually with three 5ml portions of 5% (w/v) trichloroacetic acid, followed by one 5ml portion of ethanol, and dried at 50°C. The filters were then placed in glass scintillation vials, and the precipitates dissolved by incubation for 4h at 37°C in the presence of 0.22ml of NCS solubilizer [containing 10% (v/v) water]. Toluene-based scintillation fluid (10ml) was added to each vial, and the radioactivity measured as previously described.

Product analysis by specific immunoprecipitation of either total caseins or α-lactalbumin was by using the double-antibody-precipitation technique of Von der Helm & Duesberg (1975). To a single assay, or up to three pooled assays, was added 2μl of specific antiserum followed by Nonidet P-40 to a concentration of 0.5%. Samples were then incubated for 10min at 20°C and then overnight at 4°C. Then 40–60μl of goat anti-(rabbit IgG) antiserum (the amount determined by titration to be in excess of that required to precipitate all the IgG in 2μl of rabbit serum) was added, and the samples were incubated for 10min at 20°C, and then overnight at 4°C. Next 1ml of anti-

body-wash buffer, consisting of 10mM-Hepes, pH7.5, at 4°C containing 50mM-NaCl, 1mM-EDTA and 0.5% Nonidet P-40, was added to each sample, and the immunoprecipitate sedimented by centrifugation at $3000g_{av.}$ for 15min at 4°C. The precipitate was washed twice by resuspension and sedimentation in antibody-wash buffer, and then resuspended in 2.5ml of antibody-wash buffer and layered over a 2ml cushion of 20% (w/v) sucrose containing antibody-wash buffer and centrifuged for 30min at $21000g_{av.}$ in a Beckman L2-65B ultracentrifuge in a SW50.1 rotor at 4°C. The resulting supernatant was carefully removed by using a Pasteur pipette, and the sedimented immunoprecipitate resuspended in 1ml of antibody-wash buffer and resedimented at $4000g_{av.}$ for 15min at 4°C. The final immunoprecipitates were dissolved in 0.25ml of 0.1M-NaOH, reprecipitated with 3ml of 10% (w/v) trichloroacetic acid, collected on Whatman GF/C discs, washed, dissolved in NCS solubilizer and monitored for radioactivity as described above.

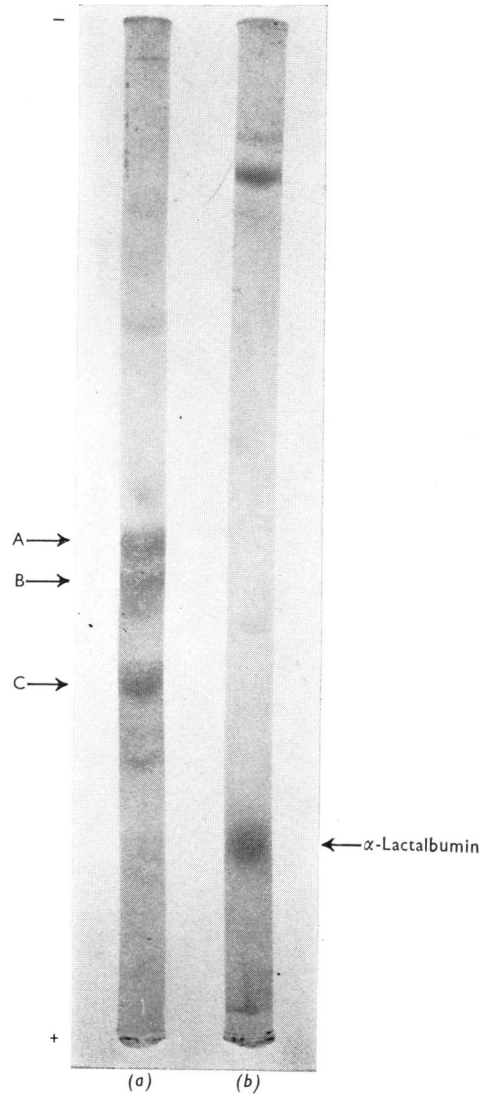
Analysis of immunoprecipitates by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was done on 10% polyacrylamide gels as described by Weber *et al.* (1972). The washed immunoprecipitates were dissolved by heating for 3min at 100°C in 80μl of 10mM-sodium phosphate buffer, pH7.2, adjusted to 2% (w/v) sodium dodecyl sulphate and 3% (v/v) 2-mercaptoethanol, in the presence of the appropriate ^{131}I-labelled internal marker protein. A trace of Bromophenol Blue tracker dye was added, and the samples were loaded in sucrose on to 11.0cm× 0.5cm gels. Electrophoresis was at 8mA/gel with circulating buffer for 6.5h. The gels were frozen at −20°C, sliced into 1.1mm lengths, and processed for radioactivity counting by using alkaline peroxide and NCS solubilizer as described by Goodman & Matzura (1971). Radioactivity data were recorded on punch-tape and processed by a ICL 1906A computer; corrections were made for overlap of ^{131}I into the ^{3}H channel, and the results plotted (as c.p.m.) such that the apparent maximum peak height of the ^{131}I-labelled internal marker protein was 0.75 that of the maximum ^{3}H-labelled peak of protein synthesized *in vitro*.

Iodination of protein. Total casein and α-lactalbumin were iodinated at 4–6 weekly intervals with either ^{125}I or ^{131}I by using the method of Hunter & Greenwood (1962). Iodinations were done in a final volume of 1.0ml containing 100mM-Hepes, pH7.6, 1mM-chloramine-T, 10μM-KI, 0.1–1.0mCi of carrier-free radioisotope and 1mg of the appropriate protein. Reactions were stopped after 1min at 20°C, and the samples dialysed exhaustively at 4°C against 5mM-Tris/HCl, pH7.5 at 4°C, containing 500mM-NaCl, and then against 5mM-Tris/HCl, pH7.5 at 4°C, alone. The iodinated proteins were stored at −20°C. Efficiency of labelling was between 20 and 30%. ^{125}I-labelled ovalbumin was a gift from Dr. A. J. Kenny of this Department.

Casein molecular-weight estimations. Casein molecular weights were estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described by Weber *et al.* (1972), by using 10% polyacrylamide gels. Gels (9.0cm×0.5cm) were allowed to polymerize overnight in the presence of one-half the standard amount of *NNN'N'*-tetramethylethylenediamine, and then pre-electrophoresed with circulating buffer for 2h at 2mA/rod immediately before use. Samples were run into the gels at 4mA/rod without circulating buffer; the current was then increased to 8mA/rod and the buffer circulated. Electrophoresis was for 5.0h at constant current with a voltage gradient of 5.8V/cm. Gels were fixed and stained overnight in a solution containing 0.05% Coomassie Brilliant Blue R, 5% (v/v) ethanol, and 12.5% (w/v) trichloroacetic acid, then destained in 12.5% (w/v) trichloroacetic acid, and stored in water.

Molecular-weight estimations were based on the comparative mobilities of the following standard proteins of known molecular weight with respect to Bromophenol Blue: bovine serum albumin (mol.wt. 68000; Tanford *et al.*, 1967); ovalbumin (hen, mol.wt. 45000; Cunningham *et al.*, 1963); lactate dehydrogenase (pig heart, mol.wt. 36000; Castellino & Barker, 1968); trypsin (bovine pancreas, mol.wt. 23300; Weber *et al.*, 1972); α-lactalbumin (guinea pig, mol.wt. 14500; Brew, 1972).

CNBr cleavage of α-lactalbumin and α-lactalbumin immunoprecipitates. ^{3}H-labelled immunoprecipitates derived from the incubation of mammary-gland mRNA in a wheat-germ system were dissolved with 10mg of carrier α-lactalbumin in 2.0ml of 0.2M-Tris/HCl, pH8.6 at 20°C, containing 8M-deionized urea and 1% (v/v) 2-mercaptoethanol. The solution was left for 4h at room temperature, aminoethylated as described by Brew & Hill (1970), dialysed exhaustively against water and finally freeze-dried. The freeze-dried protein was dissolved in 10ml of 70% (v/v) formic acid, 100mg of CNBr was added, and the resulting solution incubated at room temperature for 48h. The solution was then diluted 10-fold with water, frozen and freeze-dried. The resulting material was dissolved in 1.5ml of 5% formic acid and loaded in sucrose on a column (1.25cm×145cm, bed volume 190ml) of Sephadex G-75 (superfine grade) run under gravity at a flow rate of 2ml/h. The E_{280} of each fraction was recorded against 5% formic acid; the fractions were then transferred to glass scintillation vials, frozen and freeze-dried. Then 220μl of NCS solubilizer containing 10% (v/v) water was added to each vial, the vial capped and incubated overnight at 37°C; 10ml of toluene-based scintillation fluid was added per vial, and the ^{3}H monitored as described above.



EXPLANATION OF PLATE 1

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of guinea-pig milk proteins

Electrophoresis was performed in 10% gels as described by Weber *et al.* (1972). Protein bands were identified by staining with Coomassie Brilliant Blue. (*a*) Guinea-pig milk casein fraction (20 μg); (*b*) guinea-pig milk whey fraction (15 μg).

(*facing p.* 62)

Results

Characterization of guinea-pig milk-protein species

Unlike bovine milk proteins, which have been well characterized (see review by Jenness, 1974), the only protein of the guinea-pig milk that has been extensively characterized and its primary sequence determined is α-lactalbumin (Brew, 1972). To investigate the total mRNA fraction from a lactating mammary gland, it was necessary initially to carry out a limited analysis of the other guinea-pig milk proteins.

Milk proteins may be separated on the basis of acid precipitation at pH4.6 into two fractions, those that precipitate, the caseins, and those that remain in solution, the whey proteins. Guinea-pig milk protein is composed of about 78% caseins (6.9g/100ml) and 22% whey protein (1.9g/100ml).

Analysis of the casein fractions by electrophoresis on sodium dodecyl sulphate/10% polyacrylamide gels (Plate 1*a*) revealed the presence of three major components referred to as casein A, casein B and casein C, of estimated mol.wt. 28000, 25500 and 20500 respectively (see Fig. 1). In addition, several minor components of both higher and lower molecular weights were also present. Similar analysis of the whey fraction (Plate 1*b*) revealed two major components, the well-characterized α-lactalbumin (mol.wt. 14500) and a high-molecular-weight species. The latter is not synthesized in the lactating guinea-pig mammary gland, as sodium dodecyl sulphate/polyacrylamide-gel analysis of a [^{3}H]leucine-labelled pH4.6 soluble fraction, derived from a slice-incubation experiment (performed as described by Brew & Campbell, 1967*b*), revealed the presence of a single peak of radioactivity only, in a position corresponding to α-lactalbumin (R. Craig & O. Harrison, unpublished work). It seems likely that this protein is serum albumin, a conclusion reached by Brew & Campbell (1967*a*) on the basis of comparative electrophoretic mobilities with guinea-pig serum proteins.

Characterization of antiserum to milk proteins

Antisera raised against total casein and α-lactalbumin were tested for both potency and specificity by separate titrations of ^{125}I-labelled casein, and ^{125}I-labelled α-lactalbumin against both antisera, and also against preimmunization rabbit serum. The results (Fig. 2) demonstrate (i) a positive immune response of each antiserum against its respective antigens, (ii) the apparent lack of cross-reaction, and (iii) the lack of reaction of control preimmunization sera with either antigen.

The degree of specificity of the individual antisera was further investigated by the double-antibody-precipitation procedure of Von der Helm & Duesberg (1975) by using a mock wheat-germ assay containing small amounts of either of the following ^{125}I-labelled proteins; casein, α-lactalbumin or ovalbumin (0.25μg of ^{125}I-labelled protein containing 10^5c.p.m. added per assay). Assays were processed as described under 'Cell-free protein synthesis and product analysis'. Such analysis demonstrated that less than 1.0% of the total counts, independent of the protein added, was recovered when preimmunization serum was used; less than 1.5% of the added α-lactalbumin was precipitated by using antisera against total casein, and less than 2.0% of the casein by using antisera raised against α-lactalbumin.

Antisera to total casein contained IgG (assumed) against all the individual casein fractions. No difference could be detected between the radioactivity profiles obtained after electrophoresis of ^{125}I-labelled total casein before or after double antibody precipitation, on sodium dodecyl sulphate/10% polyacrylamide gels (profiles not shown).

Purification and characterization of mRNA

Poly(A)-containing mRNA was isolated from the post-nuclear RNA of lactating guinea pigs by affinity chromatography on oligo(dT)–cellulose. After two cycles of selective adsorption on oligo(dT)–cellulose, 0.5–0.6% of the starting material was recovered, and mRNA activity was demonstrated in both the wheat-germ and Krebs II ascites-cell-based

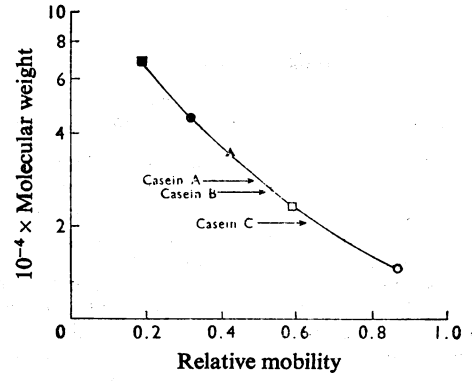


Fig. 1. *Determination of molecular weights of major casein fractions by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis*

Electrophoresis was performed in 10% polyacrylamide-gels as described by Weber *et al.* (1972). Guinea-pig caseins (20μg) and standard proteins (5μg each) were electrophoresed on parallel gels. The molecular weights of the caseins were determined by comparison of their relative mobilities, with respect to Bromophenol Blue tracker dye (mobility = 1), with those of the standard proteins of known molecular weight. Standard proteins: ■, bovine serum albumin; ●, ovalbumin; ▲, lactate dehydrogenase; □, trypsin; ○, α-lactalbumin.

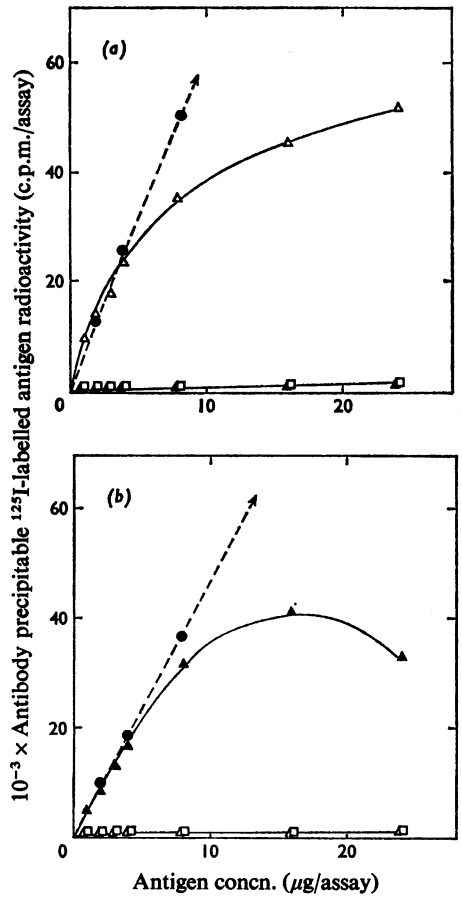


Fig. 2. *Specificity of guinea-pig anti-casein and anti-(α-lactalbumin) sera for their respective antigens*

Samples (100 μl) of sera were titrated with various amounts of ^{125}I-labelled casein (specific radioactivity 6.3×10^3 c.p.m./μg of protein) and ^{125}I-labelled α-lactalbumin (specific radioactivity 4.55×10^3 c.p.m./μg of protein) as described under 'Methods'. (*a*) Specificity of anti-casein serum as demonstrated by the ability of control serum (□), anti-(α-lactalbumin) serum (▲) and anti-casein serum (△) to precipitate casein. (*b*) Specificity of anti-(α-lactalbumin) serum as demonstrated by the ability of control serum (□), anti-(α-lactalbumin) serum (▲) and anti-casein serum (△) to precipitate α-lactalbumin. The total precipitable ^{125}I c.p.m. added to each individual assay is also represented for each antigen (●).

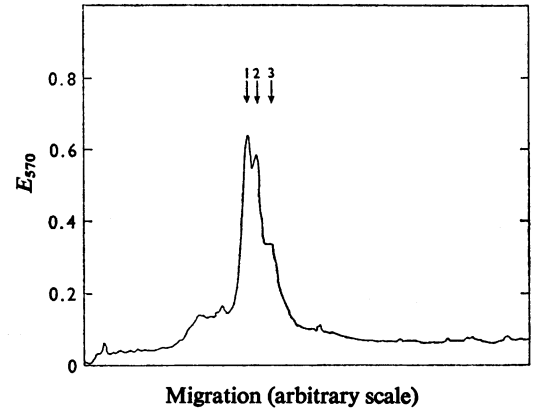


Fig. 3. *Electrophoresis of lactating-mammary-gland poly(A)-containing mRNA species in the presence of formamide*

mRNA (0.1 E_{260} unit), isolated by two cycles of affinity chromatography on oligo(dT)-cellulose, was freeze-dried, dissolved in buffered formamide and electrophoresed on 4% polyacrylamide in the presence of formamide as described under 'Methods'. Gels were stained in Stainsall, de-stained and scanned at 570 nm. For molecular-weight determinations, reticulocyte total polyribosomal RNA (0.2 E_{260} unit) was either co-electrophoresed or electrophoresed in parallel with the mammary-gland mRNA species. Arrows (1, 2 and 3) indicate the positions of the major mRNA species.

cell-free systems. Stimulation of the cell-free systems by added mammary-gland or globin mRNA varied from preparation to preparation. In the wheat-germ cell-free system, maximum protein-synthesizing activity was obtained in the presence of 0.2mM-spermidine, with a 15–50-fold stimulation over the endogenous system without mRNA added. Stimulation of the Krebs II ascites-cell system varied from 6- to 12-fold over the endogenous system. Addition of rabbit reticulocyte initiation factor-3 to the ascites-cell system resulted in a net 5–6-fold increase of the overall rate of both the endogenous and the mRNA-directed protein synthesis, when assayed under optimum conditions.

Analysis of this mRNA fraction by electrophoresis under denaturing conditions on 4% polyacrylamide gels in the presence of formamide (Fig. 3) revealed the presence of three major RNA species, and the apparent absence of rRNA species. Comparision of the mobility of these species with rabbit reticulocyte RNA markers either electrophoresed in parallel, or co-electrophoresed with the mammary-gland mRNA species, gave molecular-weight estimates of 5.4×10^5, 4.5×10^5 and 3.3×10^5 (see Fig. 4), for bands 1, 2 and 3 respectively. Slightly higher molecular-weight estimations were obtained when guinea-pig liver rRNA species were used as markers.

Product analysis of translation products of mRNA in heterologous systems

In order to estimate the total percentage of lactating-mammary-gland mRNA activity attributable to

milk proteins, mRNA was assayed in an ascites system boosted by a reticulocyte initiation factor-3 fraction, and product analysis performed with a mixture of antisera to total casein and α-lactalbumin. The results (Table 1) demonstrate that 83.5% of the total protein synthesized was precipitable by specific antibodies to milk proteins, whereas in a parallel series of assays directed by rabbit globin mRNA, only 1.8% of the total ^{3}H-labelled amino acid incorporated into protein was so precipitated. Translation of the same mRNA preparation in the wheat-germ system resulted in recovery of 89% of the total ^{3}H-labelled protein by immunoprecipitation with antibodies to milk proteins.

Investigation of the nature of the ^{3}H-labelled immunoprecipitable proteins synthesized *in vitro* was carried out by electrophoresis of the products with ^{131}I-labelled milk proteins serving as internal markers, on sodium dodecyl sulphate/10% polyacrylamide gels. Gel analysis was carried out separately on α-lactalbumin and total casein, immunoprecipitates derived from cell-free synthesis in the three systems based on wheat-germ, Krebs II ascites cells and Krebs II ascites cells stimulated by the addition of reticulocyte initiation factor-3.

(*i*) *Identification of a precursor to α-lactalbumin.* Polyacrylamide-gel analysis of immunoprecipitates derived from wheat germ (Fig. 5) revealed a single major peak of radioactivity migrating more slowly than the ^{131}I-labelled α-lactalbumin internal marker, with an estimated mol.wt. of 15500. Similar analysis of α-lactalbumin immunoprecipitates derived from the ascites cell-free system (Fig. 6), revealed two significant peaks of radioactivity, one of mol.wt. 15500 and the other which co-electrophoresed with the α-lactalbumin internal marker. Further analysis by using an ascites system stimulated by the addition of reticulocyte initiation factor-3 again showed the same two species (Fig. 7); however, the initiation factors

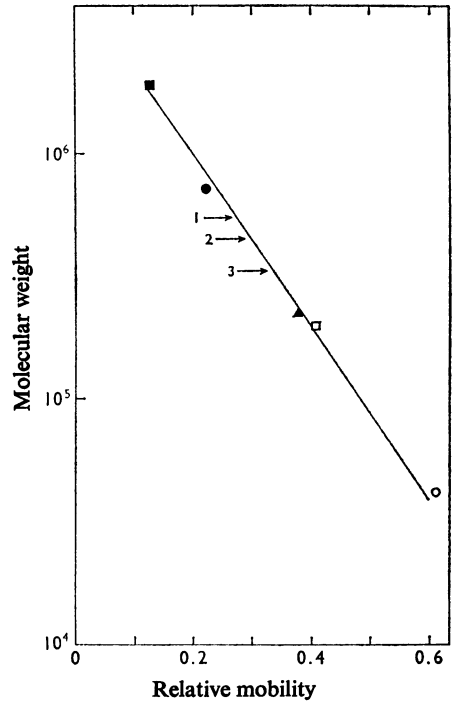


Fig. 4. *Determination of molecular weights of lactating-mammary-gland poly(A)-containing mRNA species*

Experimental techniques were as described in Fig. 3. Molecular-weight determinations were based on the relative mobilities of the guinea-pig mRNA species (arrowed 1, 2 and 3) and those of reticulocyte polyribosomal RNA species with respect to Bromophenol Blue tracker dye (mobility = 1.0). Total polyribosomal RNA (0.2 E_{260} unit) was either co-electrophoresed or electrophoresed in parallel with the mammary-gland mRNA species. RNA species: ■, 28S rRNA; ●, 18S rRNA; ▲, 10S globin mRNA; □, 9S globin mRNA; ○, 5S rRNA.

Table 1. *Measurement of the total percentage of lactating mammary-gland mRNA attributable to milk proteins*

mRNA species from rabbit reticulocytes and poly(A)-containing mRNA from guinea-pig lactating mammary gland were assayed in duplicate in a Krebs II ascites-cell system containing added reticulocyte initiation factors. Total protein synthesis and total milk-protein synthesis were determined by acid precipitation and double-antibody precipitation respectively, each on individual assays. Antibody precipitation was performed with 1:1 (v/v) mixture of rabbit antisera to α-lactalbumin and total casein.

mRNA source	Total ^{3}H incorporated into protein (c.p.m.) (*a*)	Total ^{3}H incorporated into protein: background subtracted (c.p.m.) (*b*)	Total ^{3}H immuno-precipitable (c.p.m.) (*c*)	Total ^{3}H immuno-precipitable: background subtracted (c.p.m.) (*d*)	Total protein synthesized immuno-precipitable (%) ($d/b \times 100$)
Lactating guinea pig	375000	338000	289000	282000	83.5
None	36800	—	6910	—	—
Rabbit reticulocyte	222000	185000	10000	3120	1.8

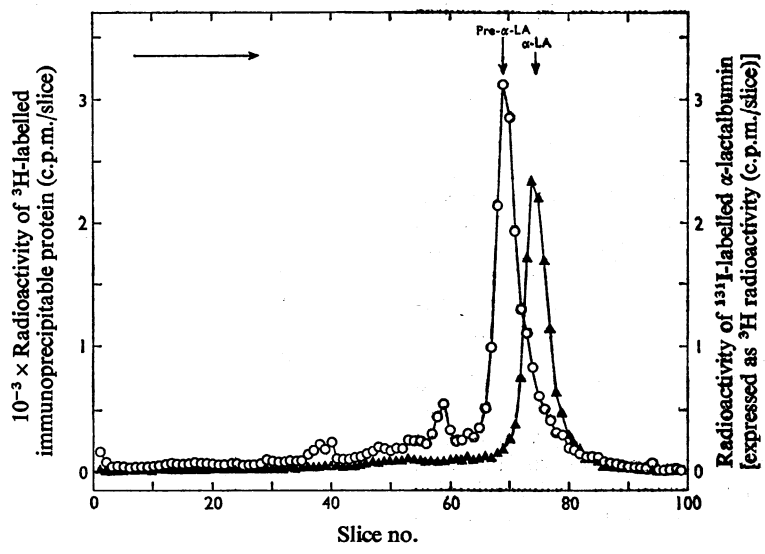


Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a ^{3}H-labelled amino acid-labelled α-lactalbumin immunoprecipitate, derived from a wheat-germ cell-free system directed by lactating-mammary-gland mRNA species*

Experimental techniques and conditions were as described under 'Methods'. Gels were sliced into 1.1mm sections. ○, Immunoprecipitated ^{3}H-labelled α-lactalbumin; ▲, ^{131}I-labelled α-lactalbumin. For the ^{131}I-labelled-protein profile, results are presented so that the maximum peak height of ^{131}I radioactivity is equal to 0.75 times the maximum peak height of the ^{3}H radioactivity. ^{131}I radioactivity (c.p.m./slice) is expressed in terms of the ^{3}H radioactivity; the necessary scaling factor in this, and other Figures, is given in the axis label. The position of ^{3}H-labelled pre-α-lactalbumin and ^{131}I-labelled α-lactalbumin have been shown by arrows labelled pre-α-LA and α-LA respectively. The horizontal arrow shows the direction of migration.

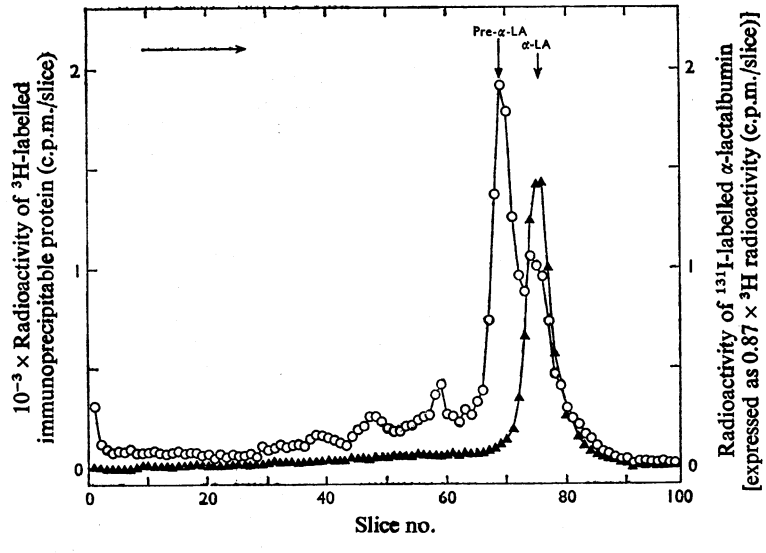


Fig. 6. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a ^{3}H-labelled amino acid-labelled α-lactalbumin immunoprecipitate, derived from a Krebs II ascites cell-free system, directed by lactating-mammary-gland mRNA species

Experimental techniques, conditions and other details were as described in Fig. 5. ○, Immunoprecipitated ^{3}H-labelled α-lactalbumin; ▲, ^{131}I-labelled α-lactalbumin.

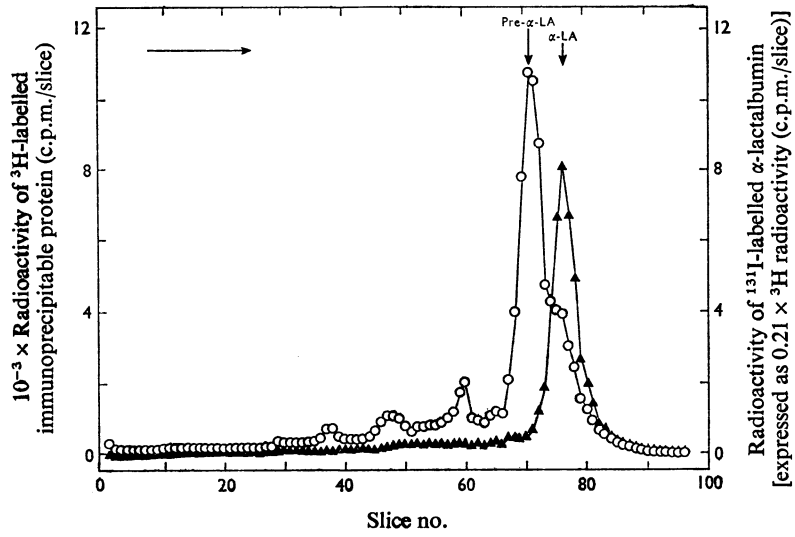


Fig.7. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of* ^{3}*H-labelled α-lactalbumin immunoprecipitate, derived from a Krebs II ascites cell-free system containing reticulocyte initiation factors, and directed by lactating-mammary-gland mRNA species*

Experimental techniques, conditions and other details were as described in Fig. 5. ○, Immunoprecipitated ^{3}H-labelled α-lactalbumin; ▲, ^{131}I-labelled α-lactalbumin.

appear to stimulate primarily the synthesis of the high-molecular-weight species. These results clearly indicate the presence of a mRNA species for α-lactalbumin in the post-nuclear mRNA preparation. In addition the results are consistent with the synthesis of α-lactalbumin *in vitro* in a precursor form. This in turn appears to undergo limited cleavage in the ascites cell-free system, yielding a molecule that co-electrophoreses with α-lactalbumin.

Analysis of all cell-free systems directed by globin mRNA, using milk-protein antisera and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, gave only a constant back-ground 'noise' of 20–60c.p.m. per slice.

This apparently specific cleavage cannot take place in the wheat-germ cell extract. The possibility exists that two mRNA species are present, one of which is not translated by the wheat germ. However, the synthesis of two electrophoretically separable protein species both immunoprecipitable by α-lactalbumin-specific antisera can only be the result of the translation of a single mRNA species, as the addition of reticulocyte initiation factors to the ascites cell-free system stimulates primarily the synthesis of the precursor molecule. This strongly suggests that the presence of the smaller species (α-lactalbumin) must depend on the presence of a specific proteolytic enzyme in the ascites S-30 fraction, which is absent from that of the wheat germ, and is not due to the existence of a separate mRNA species untranslated by the wheat germ.

We have been able to gain some insight into the nature of this modification, as guinea-pig α-lactalbumin contains a single methionine residue situated at position 90 in a total polypeptide of 123 residues. Cleavage of the molecule with CNBr results in two peptides easily separated by gel filtration (Brew, 1972). Fig. 8 shows the results of gel filtration on Sephadex G-75 (superfine grade) of α-lactalbumin after treatment with CNBr. The three peaks correspond to uncleaved α-lactalbumin, the *N*-terminal and *C*-terminal fragments. Examination of the immunoprecipitable ^{3}H-labelled profiles, derived from a wheat-germ system, cleaved in the presence of carrier α-lactalbumin, revealed four distinct peaks. The first chromatographed in the void volume and presumably contained a mixture of undissociated antibody–antigen complex along with a limited amount of casein also precipitated (see minor peaks in Figs. 5, 6 and 7). Of the remainder, one chromatographed as a single fraction ahead of the α-lactalbumin marker and must be attributable to uncleaved precursor. The next, though slightly skewed in profile, chromatographed three fractions ahead of the *N*-terminal marker, and the last peak of radioactivity co-chromatographed with the *C*-terminal marker. This not only confirmed the existence of the precursor α-lactalbumin molecule, but also provided com-

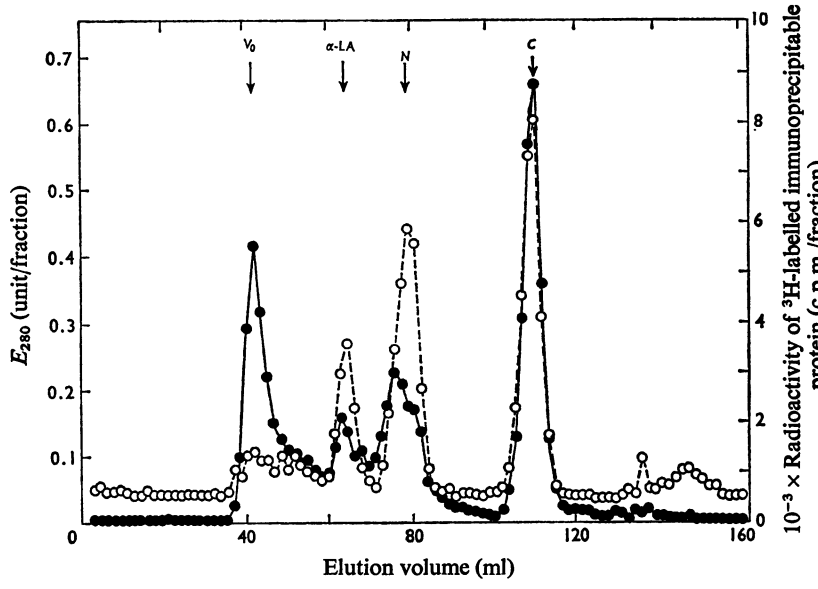


Fig. 8. *Gel filtration of peptides resulting from CNBr cleavage of [^{3}H]tyrosine-labelled α-lactalbumin immunoprecipitates*

CNBr cleavage of three pooled ^{3}H-labelled α-lactalbumin immunoprecipitates (110000c.p.m.) derived from a wheat-germ cell-free system directed by poly(A)-containing lactating-mammary-gland mRNA species, was done as described under 'Methods' in the presence of carrier α-lactalbumin (10mg). The resulting peptides were separated by gel filtration in 5% (v/v) formic acid on a column of Sephadex G-75 (superfine grade). ○, E_{280}; ●, ^{3}H-labelled immunoprecipitable peptides. V_0, void volume; *N*, *N*-terminus; *C*, *C*-terminus; α-LA, α-lactalbumin.

pelling evidence that the additional amino acid residues are situated at the *N*-terminus. The difference in molecular weight between the precursor α-lactalbumin (15500) and the α-lactalbumin (14500) is consistent with the presence of seven to ten additional amino acid residues at the *N*-terminus.

The addition of ascites S-30 extract to the precursor α-lactalbumin synthesized in a wheat-germ system (after the inhibition of protein synthesis by RNAase) did not result in the formation of α-lactalbumin.

(*ii*) *Analysis of casein immunoprecipitates.* Analysis of total casein immunoprecipitates from all three cell-free systems by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis provided a more complex profile less readily resolved than those obtained in the α-lactalbumin immunoprecipitation experiments. Electrophoresis of immunoprecipitates derived from the wheat germ (Fig. 9) revealed three well resolved peaks of activity (I, II and III) in addition to some higher- and lower-molecular-weight species. However, in contrast with the high-molecular-weight precursor α-lactalbumin, the casein species synthesized *in vitro* were overall of lower apparent molecular weight than the ^{131}I-labelled internal marker caseins. Analysis of casein immunoprecipitates derived from the ascites S-30 fraction, and ascites S-30 fraction stimulated by reticulocyte initiation-factor cell-free systems, revealed similar profiles (Figs. 10 and 11). Peaks I, II and III were again clearly resolved, along with minor species (unlabelled arrows). However, although the apparent molecular weights were the same as when synthesized in the wheat-germ cell-free system (25500, 22000 and 20000 for peaks I, II and II respectively), the relative proportions of these peaks were markedly different, peak I in particular being produced in very much greater amounts in the ascites-based systems than in the wheat germ. In addition, unlike the α-lactalbumin immunoprecipitation experiments, no significant differences could be observed between the overall casein immunoprecipitate profiles derived from ascites cells with or without the addition of reticulocyte initiation factor-3.

The discrepancy in mobility between the immunoprecipitated caseins synthesized *in vitro* and the iodinated caseins might be explained by: (i) a decrease in the mobility of the marker protein owing to iodination; (ii) incomplete translation of the mRNA species; (iii) the inability of the heterologous systems to perform post-translational modifications on the nascent polypeptides.

Careful analysis of the mobilities of caseins A, B and C, either iodinated or otherwise, demonstrated

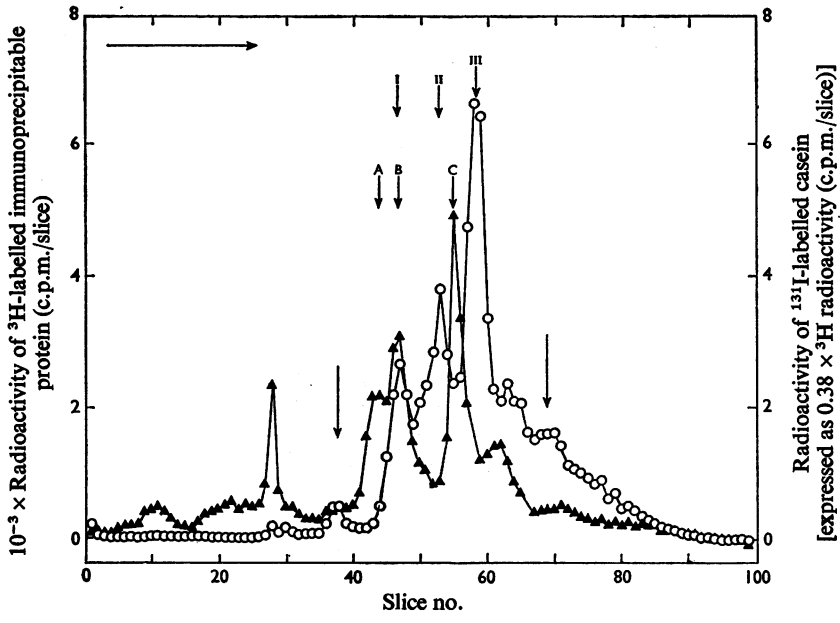


Fig. 9. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a ^{3}H-labelled total casein immunoprecipitate derived from a wheat-germ cell-free system, directed by lactating-mammary-gland mRNA species*

Experimental techniques, conditions and other details were as described in Fig. 5. ○, Immunoprecipitated ^{3}H-labelled casein; ▲, ^{131}I-labelled casein. The position of the ^{131}I-labelled marker caseins are shown by arrows labelled A, B and C; the positions of the immunoprecipitable caseins synthesized *in vitro* are shown by arrows labelled I, II and III. The two remaining arrows represent the positions of minor casein species synthesized *in vitro*, apparently not antigenically related to the major caseins.

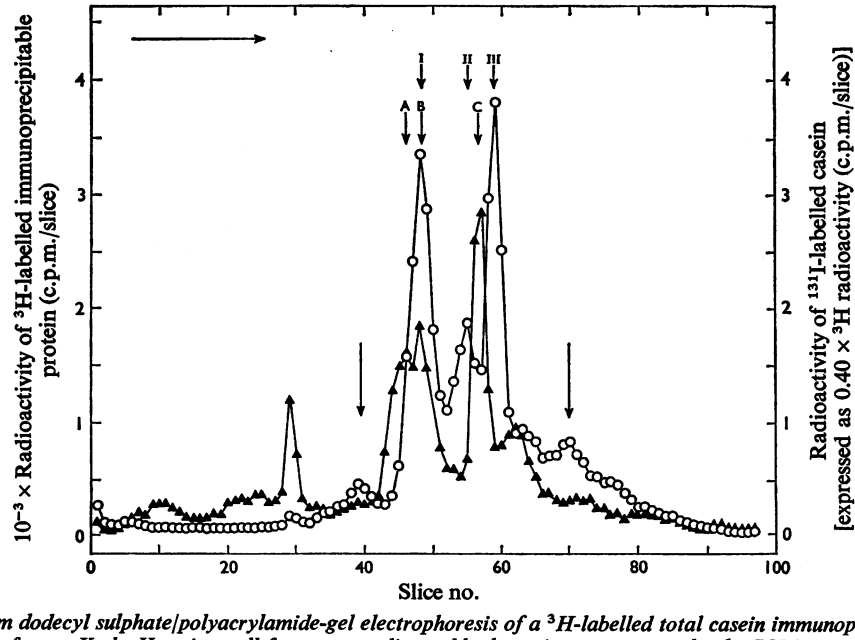


Fig. 10. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a ^{3}H-labelled total casein immunoprecipitate derived from a Krebs II ascites cell-free system, directed by lactating-mammary-gland mRNA species*

Experimental techniques, conditions and other details were described in Fig. 9. ○, Immunoprecipitable ^{3}H-labelled casein; ▲, ^{131}I-labelled casein.

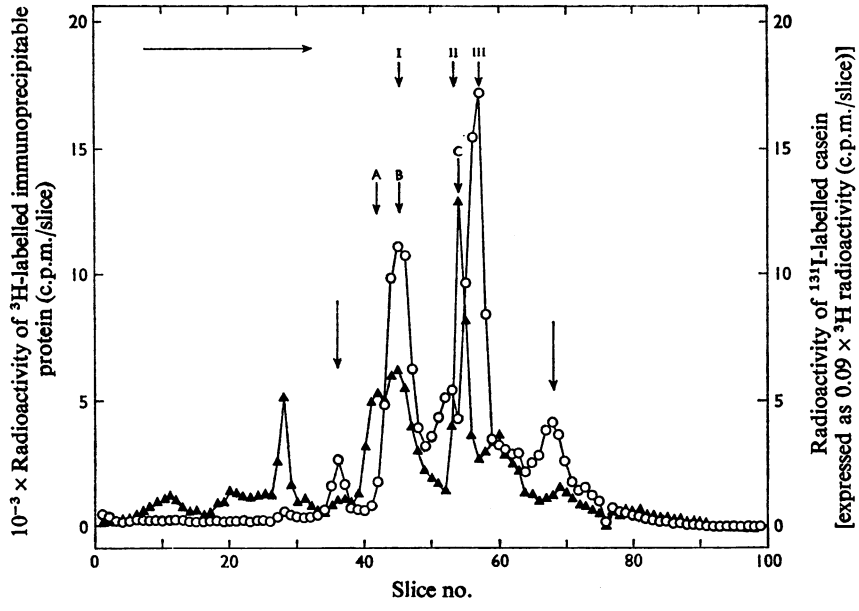


Fig. 11. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a ^{3}H-labelled total casein immunoprecipitate derived from a Krebs II ascites cell-free system containing reticulocyte initiation factors, directed by lactating-mammary-gland mRNA species*

Experimental techniques, conditions and other details were as described in Fig. 9. ○, Immunoprecipitable ^{3}H-labelled casein; ▲, ^{131}I-labelled casein.

an identical rate of migration in relation to Bromophenol Blue tracker dye. Moreover, the iodinated caseins co-electrophoresed with ^{3}H-labelled immunoprecipitates derived from incubation studies with lactating-mammary-gland slices (O. Harrison, unpublished work), clearly excluding the first possibility. The only anomaly that we cannot explain satisfactorily, with regard to the iodinated casein internal standard, is the presence of an unexpectedly large well-defined peak of radioactivity corresponding to the position of a minor high-molecular-weight casein component (see slice no. 28, in Figs. 9, 10 and 11). We can only assume that this protein is particularly susceptible to iodination, as apart from this, the iodinated casein profile is remarkably similar to that observed by standard staining techniques. The second possiblity, incomplete translation of the casein mRNA species, also appears unlikely, especially as the peaks of protein synthesized *in vitro* are well defined and of apparently identical molecular weight irrespective of the heterologous system used. Moreover, similar heterologous systems have been used with high efficiency to translate mRNA coding for relatively high-molecular-weight proteins (Rosen *et al.*, 1975*a*,*b*; Schutz *et al.*, 1974; Von der Helm & Duesberg, 1975). Concerning the third possibility, there is little to suggest that heterologous systems are capable of performing post-translational modifications other than peptide cleavage (see the Discussion section). Caseins are known to be both glycosylated and phosphorylated (Jenness, 1974). Such modifications are known to affect the mobility of peptides on sodium dodecyl sulphate/polyacrylamide gels (Banker & Cotman, 1972; Weber *et al.*, 1972). We have now separated the major guinea-pig casein fractions and preliminary data confirm that all three major components are phosphorylated and to some extent glycosylated (R. Craig, D. McIlreavy & R. Hall, unpublished work). In the light of these results, we consider it reasonable to conclude that the immunoprecipitable caseins synthesized *in vitro* in the absence of major post-translational modifying events, do in fact represent the primary translational products, giving the estimated molecular weights described above for peaks I, II and III. Data describing the apparent increased mobility of polypeptides synthesized *in vitro* compared with those synthesized *in vivo*, on sodium dodecyl sulphate/polyacrylamide gels have previously been described for immunoglobulin heavy chain synthesized in a reticulocyte lysate (Cowan & Milstein, 1973), vesicular-stomatitis-virus G protein synthesized in wheat germ (Both *et*

al., 1975) and Adenovirus 2 Fibre protein synthesized in a mammalian cell-free system based on Krebs II ascites-cell ribosomes and pH5 fraction, and rabbit reticulocyte initiation factors (Anderson *et al.*, 1974). Experiments using total casein antisera, absorbed with various combinations of the purified caseins, demonstrate that caseins A, B and C correspond to the synthesized ^{3}H-labelled peaks I, II and III *in vitro* respectively, and that the predominant minor species are not antigenically related to the major caseins or α-lactalbumin (R. Craig, unpublished work). Whether or not these synthesized peptides *in vitro* represent precursor polypeptide forms of the caseins remains as yet undetermined. There clearly exists a marked difference in the proportions of the individual casein species synthesized by the wheat-germ and the ascites-cell systems from the same mRNA preparation. Whether this is due to the presence or absence of specific proteinases from either system, or is simply an indication that the wheat-germ system in our hands operates at a lower efficiency on the higher-molecular-weight mRNA species, is clearly open to further investigation. No significant difference was observed between profiles labelled by [^{3}H]tyrosine or ^{3}H-labelled amino acid mixture for casein, in either the wheat-germ or ascites-cell-based systems.

Discussion

Guinea-pig milk, on the basis of acid precipitation and separation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, contains four major protein components synthesized by the mammary gland, three acid-precipitable caseins, and a single whey protein, α-lactalbumin.

The identification of three major caseins was not unexpected, as similar components have been identified and characterized from bovine and ewe milk (see Jenness, 1974), and from rat milk (Rosen *et al.*, 1975*a*). However, until the individual guinea-pig caseins have been fully characterized we cannot attempt to correlate them with the α, β and K components of bovine and ewe milk.

Species of mRNA coding for milk proteins have been isolated and partially characterized from the lactating mammary gland of the rat (Rosen *et al.*, 1975*a*) and the ewe (Gaye & Houdebine, 1975) by using a combination of sizing techniques and selective adsorption of poly(A)-containing RNA on nitrocellulose filters, oligo(dT)–cellulose or poly(U)–Sepharose. By two cycles of selective adsorption on oligo(dT)–cellulose we have obtained a biologically active mRNA fraction from the post-nuclear fraction of the guinea-pig lactating mammary gland. Analysis of this by electrophoresis on 4% polyacrylamide gels in the presence of formamide shows it to be essentially free of contaminating RNA species and to consist of three predominant RNA bands, two major and one minor, a situation very similar to that reported for rat milk-protein mRNA species.

Translation of the mRNA species in cell-free systems, followed by immunoprecipitation, revealed that in both the wheat-germ and ascites systems more than 80% of the total acid-precipitable ^{3}H-labelled protein was in the form of milk proteins. The amount of total casein and α-lactalbumin respectively could not be assessed with precision, as although single-antibody titrations, and double-antibody-precipitation experiments using high-specific-radioactivity ^{125}I-labelled antigens, revealed essentially no cross-reaction, the gel profiles (Figs. 5, 6 and 7) clearly indicated a low extent of cross-reaction between antibody raised against α-lactalbumin and the casein fractions. However, cross-reaction between α-lactalbumin and antiserum to total casein appeared to be negligible. Essentially no difference in the profiles of the gel electrophoresis of immunoprecipitable caseins could be detected by using antiserum to total casein either adsorbed or otherwise with α-lactalbumin.

On the basis of these observations, caseins represented 65–75% of the total protein synthesized in all three heterologous cell-free systems. Such observations emphasize the extreme sensitivity of the double-antibody technique compared with conventional methods requiring the addition of carrier antigen and large quantities of antiserum. This technique is particularly useful, as guinea-pig milk proteins, like those of the rat, proved to be particularly weak antigens (Rosen *et al.*, 1975*a*). In addition, recoveries are essentially quantitative, and do not require correction for losses, presumably in the form of soluble complexes, as reported elsewhere when single-antibody techniques were used (Rosen *et al.*, 1975*a*,*b*; Houdebine & Gaye, 1975*a*).

Analysis of α-lactalbumin immunoprecipitates provide compelling evidence, not only that the total mRNA fraction contains mRNA specific for α-lactalbumin, but that this is synthesized *in vitro* in a precursor form, modified at the *N*-terminus. In addition the wheat-germ system lacks the specific endopeptidase, present in a limited amount in the ascites system, which is capable of cleaving the precursor to a form which co-migrates with ^{131}I-labelled α-lactalbumin. The synthesis by the wheat-germ cell-free system of precursor protein molecules which have not been previously detected in cell extracts is not unique. Kemper *et al.* (1974) reported the synthesis of the pre-proparathyroid hormone by a wheat-germ cell-free system, and later demonstrated a similar result to our own, in that the same mRNA fraction translated in an ascites-cell system resulted in the synthesis of both the pre-proparathyroid hormone and the cleavage product, proparathyroid hormone (Habener *et al.*, 1975). Similar results, in which the authentic peptide has been identified after

synthesis in the ascites-cell system, whereas translation of the same mRNA in the wheat-germ system has resulted in the production of a larger peptide, have been reported for pituitary growth hormone (Bancroft *et al.*, 1973; Sussman *et al.*, 1976) and human placental lactogen (Boime *et al.*, 1975).

The processing of translational products *in vitro* was first reported by Milstein *et al.* (1972), who demonstrated the synthesis in response to added mRNA of a precursor immunoglobulin light chain modified at the *N*-terminus in a reticulocyte lysate, but that when translation occurred in an ascites S-30 system, only the processed light chain could be detected. On the basis of these results, it was proposed that the ascites system contained a limited amount of membrane material that was responsible for this cleavage, the lack of membrane in the reticulocyte system being reflected by the absence of cleavage. This work has been elegantly extended by Blobel & Dobberstein (1975*a*,*b*), who report that the specific cleavage of immunoglobulin light chain is a membrane function, and occurs only during ongoing translation of the nascent peptide. In the light of these observations, and others concerning *N*-terminal sequence homology of putative precursors to pancreatic secretory proteins (Devillers-Thiery *et al.*, 1975), the 'signal' hypothesis has been proposed (Blobel & Dobberstein, 1975*a*), which in essence postulates the existence of a metabolically short-lived *N*-terminal extension of all proteins to be segregated in membrane-bound compartments. Such an extension would result from the translation of an additional sequence of codons at the 5′ end of the mRNA, between the initiation codon and the codon specifying the *N*-terminal amino acid of the functional 'cleaved' protein.

Milk proteins, undoubtedly secretory proteins, are synthesized primarily, if not exclusively, on membrane-bound polyribosomes (Houdebine & Gaye, 1975*b*; Harrison *et al.*, 1976), and consequently should make ideal candidates with which to test the 'signal' hypothesis. We have demonstrated *in vitro* the existence of a labile precursor α-lactalbumin molecule (pre-α-lactalbumin). The apparent inability of the ascites-cell S-30 fraction to cleave pre-α-lactalbumin synthesized in a wheat-germ system is in agreement with the signal hypothesis, as cleavage appears to be a function of ongoing translation. Presumably pre-α-lactalbumin is synthesized on free polyribosomes both in the wheat-germ and ascites systems, but the cleavage occurs only during synthesis on bound polyribosomes in the ascites cell-free system. However, unlike the putative precursors in dog pancreas, the immunoglobin light-chain precursor and pre-proparathyroid hormone, which possess an estimated additional 20–25 amino acid residues at the *N*-terminus, pre-α-lactalbumin according to molecular-weight estimates has only an additional seven to ten amino acid residues at the *N*-terminus. This is more analogous to the additional hexapeptide present at the *N*-terminus of pro-albumin (Quinn *et al.*, 1975; Russell & Geller, 1975). The significance of pro-albumin in terms of the 'signal' hypothesis remains to be clarified.

The absence of antiserum specific to the individual guinea-pig caseins, and the difference in electrophoretic mobilities between caseins synthesized *in vivo* labelled with ^{131}I and immunoprecipitable products synthesized *in vitro*, clearly complicate the detailed analysis of any casein precursor–product relationship. Direct comparison of the gel profiles of wheat-germ and ascites-cell synthesized casein immunoprecipitates points to the absence of a large (20–25 amino acid) precursor polypeptide for the caseins. However, this does not exclude the presence of a smaller peptide addition similar to that observed with α-lactalbumin. Gaye & Houdebine (1975), using the reticulocyte lysate (which synthesizes a precursor to immunoglobulin light chain) to translate ewe milk-protein mRNA species, have demonstrated by specific antibody precipitation and subsequent sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the synthesis of β-lactoglobulin, α_S-, β- and K-casein as single protein peaks, each co-electrophoresing with labelled polypeptides isolated from mammary explants incubated *in vitro*. α-Lactalbumin synthesis was also detected, but no gel profiles were presented. No evidence was found which might suggest the existence of precursor molecules, though ewe caseins synthesized *in vitro* by reticulocyte lysates co-migrate with the internal markers, which presumably are both phosphorylated and glycosylated (Soulier *et al.*, 1975), in sharp contrast with our own results. The gel data of Rosen *et al.* (1975*a*) on rat caseins is less definitive. Sodium dodecyl sulphate/polyacrylamide-gel analysis of an immunoprecipitate resulting from the addition of a mixture of antisera to all three major rat caseins to a wheat-germ system resulted in a single peak of activity migrating between the casein markers of mol.wt. 25000 (rat casein 3) and 30000 (rat casein 2). Some material of lower molecular weight and a small peak co-migrating with the rat casein 1, of mol.wt. 42000, were also observed. It remains to be demonstrated whether the major rat immunoprecipitable casein band synthesized *in vitro* corresponds to casein band 2, indicating that increased mobility may be equated with the absence of post-translational modifications, or alternatively whether it corresponds to a relatively large precursor to rat casein 3.

Calculations concerning the possible coding capacity of mRNA species are only approximate at best. mRNA molecular-weight estimates based on electron micrographs of the denatured spread mRNA appear to be the most reliable (Lizardi *et al.*, 1975; Woo *et al.*, 1975). Size estimations by electro-

Table 2. *Coding potential of lactating-mammary-gland mRNA species*

	Estimated subunit mol.wt.		Estimated no. of amino acids	
	in vivo	*in vitro*	*in vivo*	*in vitro*
Casein A	28000	25500	234	213
Casein B	25500	22000	213	183
Casein C	20500	20000	171	167
α-Lactalbumin	14500	15500	123 (known) *	133
Lactating-mammary-gland mRNA	Apparent mol.wt.		Estimated coding capacity (no. of amino acids)	
Band 1	5.3×10^5		500	
Band 2	4.6×10^5		430	
Band 3	3.2×10^5		290	

* Brew (1972).

phoresis or gradient centrifugation are particularly prone to variations in RNA secondary structure, this affecting the true mobilities of both mRNA and rRNA marker species. Woo *et al.* (1975) have reported, in experiments involving ovalbumin mRNA, that gel electrophoresis in the presence of 99% formamide results in an over-estimation of molecular weight, probably owing to incomplete denaturation of the 28S and 18S rRNA marker species.

Calculations based on mRNA molecular weights derived by gel electrophoresis in the presence of formamide, allowing for an average poly(A) tail size of 90 nucleotides (Table 2), demonstrate with great clarity that the coding capacity of the predominant guinea-pig mRNA species is far in excess of that required to code for any single guinea-pig milk protein, even when allowing for possible over-estimation of true polypeptide molecular weight owing to glycosylation, and/or phosphorylation. A similar conclusion has been reached by Gaye & Houdebine (1975), who estimated the potential coding capacity of ewe mRNA species, based on the average apparent mRNA molecular weight, of the predominant species.

Thompson & Farrell (1974) have proposed that the caseins might be synthesized as a single macromolecule, subsequently cleaved into functional proteins by specific endopeptidases. Although clearly none of the major guinea-pig mRNA species contains sufficient coding capacity for all the guinea-pig caseins, we cannot as yet exclude the possibility that a single mRNA molecule might code for more than one protein. Translation might then either take place from a single initiation site, with subsequent post-translational modification of a poly-cistronic precursor protein, as with the picornaviruses (Smith, 1973; Villa-Komaroff *et al.*, 1975) and vitellogenin (Deeley *et al.*, 1975), or alternatively from multiple initiation sites, as with prokaryotic polycistronic mRNA molecules (see Stavis & August, 1970).

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