Studies on the Biosynthesis of Protein by Lactating Guinea-Pig Mammary Gland

CHARACTERISTICS OF THE SYNTHESIS OF α -LACTALBUMIN AND TOTAL PROTEIN BY SLICES AND CELL-FREE SYSTEMS

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1. Slices of lactating guinea-pig mammary gland were incubated with radioactive amino acids and the various subcellular fractions separated by centrifugation after disruption of the cells by mincing and homogenization. The most active fraction for protein synthesis appeared to be the 'mitochondrial'. 2. When the subcellular fractions were prepared without previous incubation of the cells and were then incubated with radioactive amino acid and an energy-generating system, the 'mitochondrial fraction' was at least as active for protein synthesis as the 'microsomal fraction'. 3. The ribosomes in the microsomal fraction are mainly unattached to membrane whereas those in the mitochondrial fraction are probably attached to fragments of the rough-surfaced endoplasmic reticulum. This latter fraction contains few mitochondria. 4. The combined mitochondrial and microsomal fractions incorporated radioactive amino acids into α -lactalbumin. 5. The radioactive leucine isolated from tryptic and chymotryptic peptides of α -lactalbumin synthesized in the cell-free system was not of uniform specific radioactivity. This was consistent with the polypeptide being assembled by the sequential addition of amino acids. 6. Evidence is presented for the polypeptide chain of α -lactalbumin being assembled from the *N*-terminus and for chain initiation in the cell-free system. 7. It is concluded that cell-free extracts of lactating mammary gland synthesize α -lactalbumin.

Various features of the mechanism of synthesis of milk protein have been studied previously. The determination of arteriovenous differences and the fate of injected radioactive precursors in the intact lactating animals have shown that the free amino acids of the blood are the precursors of the milk proteins (see review by Barry, 1961). With slices and single-cell suspensions derived from lactating guinea-pig mammary gland Turba & Hilpert (1961*b*) showed that free amino acids were rapidly taken up by the cells and incorporated into whey protein. That subcellular fractions also possess activity was shown by Fraser & Gutfreund (1958), who incubated a homogenate of lactating guinea-pig mammary gland in the presence of radioactive amino acid and then fractionated the homogenate. They found that the protein with the highest radioactivity was present in the 'mitochondrial' rather than the 'microsomal' fraction. That the activity of the heterogeneous mitochondrial frac-

tion was probably due to microsomal contamination was suggested by the fact that the incorporation of radioactive amino acid into protein was inhibited by very low concentrations of ribonuclease whereas, at least in liver, protein synthesis by mitochondria is insensitive to ribonuclease. Turba & Hilpert (1961*a*) later showed that the isolated microsome fraction from lactating guinea-pig mammary gland incorporated amino acid into protein by an ATP-dependent mechanism. It has been shown also that homogenates of mammary gland catalyse an ATP-dependent amino acid activation and transfer to soluble RNA (Fraser, Shimizu & Gutfreund, 1959; Bucovaz & Davis, 1961). These results suggest that the mechanism of protein synthesis in mammary tissue is similar to that in the mammalian tissues that have been studied hitherto.

During the initiation of lactation, synthesis of milk protein in the mammary gland begins and rises to a rate which has been estimated to be greater than 9000 molecules/cell/sec. (Turba & Hilpert, 1961*a*). Therefore the mammary gland is a particularly suitable organ in which to study the

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mechanism and control of mammalian protein synthesis. The aim of the present work was to develop a cell-free system from mammary gland which synthesized a specific milk protein. This would be an essential property of any system that might later be used to study the control of synthesis of milk protein. Some aspects of this work have been previously reported in a preliminary form (Brew & Campbell, 1965).

MATERIALS AND METHODS

Chemicals. The disodium salt of ATP and the sodium salt of GTP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and pyruvate kinase from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The silver-barium salt of phosphoenolpyruvic acid was prepared by the method of Clark & Kirby (1963).

Solutions of free phosphoenolpyruvic acid were prepared before use and adjusted to pH 7.4 (glass electrode) with N-KOH . Tris ('specially purified') was obtained from British Drug Houses Ltd., Poole, Dorset, and polyuridylic acid from Miles Laboratories, Stoke Poges, Slough, Bucks.

Radioactive chemicals. L-[U- ^{14}C]Leucine (specific radioactivities 4.5 mc/m-mole, 45 mc/m-mole and 198 mc/m-mole), L-[U- ^{14}C]phenylalanine (62 mc/m-mole), L-[U- ^{14}C]lysine (136 mc/m-mole), ^{14}C -labelled algal-protein hydrolysate (1 mc/5 mg.), DL-[4,5- $^3\text{H}_2$]lysine (5.0 c/m-mole), L-[4,5- $^3\text{H}_2$]lysine (8.3 c/m-mole), [^3H]hexadecane and [^{14}C]hexadecane were purchased from The Radiochemical Centre, Amersham, Bucks.

Proteins. Trypsin (batch 763) and carboxypeptidase A-DFP (batch 6131) were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A., lysozyme from British Drug Houses Ltd., Poole, Dorset, chymotrypsin (batch 36) from Seravac Laboratories (Pty.) Ltd., Holyport, Maidenhead, Berks., horse-heart cytochrome *c* from Sigma Chemical Co., and ovalbumin from Armour Pharmaceutical Co., Eastbourne, Sussex.

Scintillation chemicals. 2,5-Diphenyloxazole, 1,4-bis-(5-phenyloxazol-2-yl)benzene and naphthalene of scintillation grade were obtained from Nash and Thompson Ltd., Tolworth, Surbiton, Surrey, and Aerosil standard silica from Busch, Beach and Segner Bayley Ltd., London, E.C. 3.

Other materials. Sephadex G-25 (fine) and G-100 were purchased from Pharmacia, Uppsala, Sweden, CM-cellulose from Serva, Heidelberg, Germany, and Zeo-Karb 225 from The Permutit Co. Ltd., London, W. 1.

Starch hydrolysed for starch-gel electrophoresis (lot 201-1) was obtained from Connaught Medical Research Lab., Toronto, Canada, and cellulose acetate membranes from Oxoid Division of Oxo Ltd., London, E.C. 4.

Other chemicals were purchased from British Drug Houses Ltd. and were AnalaR grade wherever possible. Iodoacetic acid was recrystallized twice from light petroleum, and orcinol once from benzene before use.

Preparation and incubation of slices. Slices of tissue were cut by hand and incubated in a salt medium similar to that described by Anfinsen, Beloff, Hastings & Solomon (1947). The medium contained CaCl_2 (10 mM), NaCl (32 mM), KCl (33 mM), KHCO_3 (40 mM) and 0.2% glucose in twice-glass-distilled water and was equilibrated before

use with $\text{O}_2 + \text{CO}_2$ (95:5) at 25°. Medium to a volume of 5 ml. was used for each gram of slices. Incubation was carried out under $\text{O}_2 + \text{CO}_2$ (95:5) at 37°.

Homogenization of mammary tissue. Even with guinea-pig mammary gland the amount of connective tissue present renders the sole use of a glass homogenizer impractical. Therefore the mammary tissue from each animal was placed in the barrel of a tissue press (purchased from Climplex Ltd., London, N.W. 7; made to the design of Porterfield, 1960) which had been cooled previously to 0°. The plunger was tightened and portions of the extracted fine-minced tissue were collected. The tissue mince (4.0 g. portion) was then suspended in 10 ml. of medium A (10 mM- MgCl_2 , 25 mM- KCl , 35 mM-tris buffer, pH 7.8 at 25°, 0.15 M-sucrose; all solutions made up in twice-glass-distilled water; Rendi & Hultin, 1960) and further disrupted in an all-glass Potter-type homogenizer with a loosely fitting pestle.

Isolation of subcellular fractions. The following arbitrary definitions have been used for centrifugal fractionation of homogenates: nuclear fraction (including cell debris), that sedimenting at 800 g/10 min. (Spinco no. 30 rotor); mitochondrial fraction, the fraction sedimenting at 11500 g/10 min. (Spinco no. 30 rotor) from the 800 g supernatant; microsomal fraction, that sedimenting at 105000 g/60 min. (Spinco no. 40 rotor) from the 11500 g supernatant, and cell sap, the 105000 g supernatant. The mitochondrial plus microsomal fraction was that sedimenting at 105000 g/60 min. (Spinco no. 40 rotor) from the 800 g supernatant.

The microsomal or mitochondrial fractions from each 4 g. of minced mammary tissue were suspended in 1.5 ml. of medium A and the mitochondrial plus microsomal fraction in 2.0 ml. of medium A by using a small all-glass Potter homogenizer.

Treatment of cell sap by gel filtration. Up to 14 ml. of cell sap was placed on a 35 cm. $\times$ 2.5 cm. column of Sephadex G-25 equilibrated with medium A at 2° and eluted with medium A, slight N_2 pressure being used when necessary. The coloured cell sap was collected and, as judged by the intensity of colour, suffered little or no dilution during the treatment.

Incubation of subcellular fractions. Suspended particles were incubated in the presence of cell sap in air at 37°. The components of the incubation mixture are given for each experiment in the legends to Tables and Figures. After incubation the particulate fraction was recovered when required by dilution with an equal volume of medium A and centrifuged at 105000 g for 60 min.

Separation of free and bound ribosomes. The microsome fraction was subjected to sucrose density-gradient centrifugation according to the method of Henshaw, Bojarski & Hiatt (1963).

Treatment of particles. Re-isolated particles were suspended in 0.9% NaCl and treated with ultrasonic vibrations, freezing and thawing and the remaining particulate material was removed by centrifuging at 105000 g for 60 min. as described by Campbell & Kernot (1962) for rat-liver microsomes.

Isolation of total protein. Total protein was isolated by the modified Schneider procedure of Zamecnik, Loftfield, Stephenson & Steele (1951).

Isolation of α -lactalbumin. (1) From slices of tissue. In all cases casein was first removed by adjusting the pH to 4.5 with N -acetic acid and centrifuging at 800 g for 5 min., the supernatant being termed whey.

In the experiment when α -lactalbumin was isolated after different time-intervals, the whey was concentrated by placing in a dialysis sac and leaving in solid sucrose for 16 hr. The concentrated solution (approx. 2 ml.) was applied to an 85 cm. $\times$ 2 cm. column of Sephadex G-100, equilibrated with 0.1 M-ammonium acetate-0.4 M-NaCl. Elution patterns similar to those for normal guinea-pig whey were obtained (Brew & Campbell, 1967) and the α -lactalbumin peak was precipitated by adding trichloroacetic acid to 5%, collecting the protein by centrifuging at 8000g/5 min., washing with 5% trichloroacetic acid and dissolving in a small volume of water.

The α -lactalbumin content of this solution was determined by taking 0.1 ml., adding 2.9 ml. of 0.01 N-HCl and measuring the extinction at 280 m μ . The amount of the protein was determined by using $E_{280}^{1\%1\text{cm}}$ 16.7 (Brew & Campbell, 1967). Another portion of solution (0.1 ml.) was taken for determination of radioactivity.

In the experiments when α -lactalbumin labelled with [^{14}C]leucine was prepared, the same procedure was followed for removing casein and the whey dialysed against frequently changed distilled water and freeze-dried.

(2) From extracts obtained after ultrasonic disintegration. Either carrier milk (0.2 ml.) or whey was added to the extracts, casein removed as described in (1) above, the supernatants were dialysed against distilled water and freeze-dried. α -Lactalbumin was isolated by ion-exchange chromatography on CM-cellulose and the peak fractions were dialysed against distilled water and freeze-dried. The product was further purified by gel filtration on Sephadex G-100.

Preparation of antisera and precipitation of specific whey proteins with antibody. Antisera were prepared by injecting rabbits with the proteins prepared from guinea-pig whey by extraction with 0.03 N-HCl in 85% ethanol (Brew & Campbell, 1967, as described by Campbell, Greengard & Kernot, 1960). Antisera to ovalbumin were similarly prepared. When appropriate the specific radioactivity of antibody-antigen precipitates was determined as previously described.

Determination of the specific radioactivities of N-terminal and 'internal' lysine residues of α -lactalbumin. The preparation and hydrolysis of the DNP derivative of guinea-pig α -lactalbumin was accomplished as described by Brew & Campbell (1967). The N-terminal lysine (di-DNP-lysine) was isolated from the ether extract of the hydrolysate after removal of dinitrophenol by sublimation at 60° on to a cold finger, by descending chromatography on phthalate-buffered Whatman no. 1 paper by using the pentan-1-ol, pH 6.0, system of Pairent & Williamson (1960). The internal lysine (ϵ -DNP-lysine) was recovered from the aqueous phase by chromatography on phthalate-buffered Whatman no. 4 paper by using the 2-methylbutan-2-ol-phthalate system of Blackburn & Lowther (1951).

The DNP-amino acids were extracted from the paper with acetone (4 ml.) containing 1 drop of conc. HCl, the extracts evaporated to dryness and the di-DNP-lysine dissolved in 2.0 ml. of ether and the ϵ -DNP-lysine in 2.0 ml. of methanol (both at 0°). A portion of each solution was evaporated to dryness and dissolved in 3.0 ml. of 1% NaHCO₃. The amounts of the amino acid derivatives were determined from the extinction of these solutions at 355 m μ (for di-DNP-lysine) or 362 m μ (for ϵ -DNP-lysine), using the m-molar extinction coefficients of 30.0 and 17.4 reported

by Fraenkel-Conrat, Harris & Levy (1955). The remainder of the solution was evaporated on a 7 cm.² aluminium planchet at 25° (ether) or 50° (methanol) and the radioactivity determined by gas-flow counting.

Reduction and carboxymethylation of α -lactalbumin. The method of Merigan, Dreyer & Berger (1963) was used.

Oxidation of α -lactalbumin with performic acid. The method of Hirs (1956) was used.

Digestion with carboxypeptidase A. Digestion of α -lactalbumin was carried out with 1% of its weight of carboxypeptidase A-DFP for 19 hr. as described by Brew & Campbell (1967).

Digestion with trypsin or chymotrypsin. Digestion with each of these enzymes was carried out as described by Sargent & Campbell (1965) for trypsin.

Separation of peptides. (1) By high-voltage electrophoresis. Peptides from less than 1 mg. of α -lactalbumin dissolved in about 10 μ l. of 1% triethylamine were applied as a 1 cm. band 13 cm. along a 57 cm. $\times$ 5 cm. strip of Whatman no. 3MM paper. High-voltage electrophoresis was carried out in pyridine acetate buffer, pH 3.5, as described by Sargent & Campbell (1965), by using 2500 v for 60 min. After drying, the peptides were either detected by dipping in 0.4% (w/v) ninhydrin in acetone and developing at 40–50°, or 1 cm.-wide segments of the strip were cut off and eluted by placing in 5 ml. of 5% (v/v) pyridine for 24 hr. at room temperature. The paper segments were then removed, the solvent was evaporated at 70° and the radioactivity of the eluted material determined.

(2) By 'fingerprinting'. Peptides from 1–2 mg. of α -lactalbumin dissolved in 10–20 μ l. of 1% triethylamine were applied as a 1 cm. band 13 cm. along and 3 cm. from the long edge of a 57 cm. $\times$ 30 cm. piece of Whatman no. 3MM paper. High-voltage electrophoresis was carried out as described above, but with 1000 v for 180 min. After drying at 40–50° for several hours, ascending chromatography with 3-methylbutan-1-ol-pyridine-water (35:35:30, by vol.) was carried out for 16 hr., during which time the solvent front reached the top of the paper.

The peptides were detected with ninhydrin as described above and the positive regions marked with pencil. Excess of ninhydrin was removed by washing three times with acetone and the paper dried. The marked regions were cut out and eluted with 5 ml. of 5 M-ammonia or 5% (v/v) pyridine at 25° for 25 hr., the pieces of paper removed and the solvent was evaporated at 70°.

Determination of radioactivity. (1) ^{14}C alone. Protein or peptides were dissolved in formic acid and plated at infinite thinness or dried protein was plated at infinite thickness as described by Campbell *et al.* (1960). DNP-amino acids were plated by evaporating their ether or methanol solutions on 7 cm.² aluminium planchets. All samples were counted with an automatic gas-flow counter (Nuclear-Chicago model C110B) with an efficiency of 22% (Sargent & Campbell, 1965).

(2) ^3H alone or ^3H and ^{14}C . The radioactivity of peptides and proteins containing ^3H was determined in a liquid-scintillation spectrometer (Packard Tri-Carb model 314 EX) as described by Sargent & Campbell (1965).

Estimations. Protein was determined by the Folin-Ciocalteu method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. RNA was determined by the orcinol method of Mejbaum (1939), with hydrolysed yeast RNA as standard.

RESULTS

Protein synthesis by slices of lactating guinea-pig mammary gland. The time-course of incorporation of [^{14}C]lysine into protein fractions during the incubation of slices of mammary gland is shown in Fig. 1. This indicates that the 'mitochondrial' fraction is the most active for protein synthesis in the intact cell as the incorporation of radioactive amino acid into this fraction is both faster and reaches a higher value than that of the other fractions. Although there is a delay of 15–20 min. before the appearance of radioactivity in the supernatant casein and α -lactalbumin the labelling of cell-sap protein shows no such lag phase.

Characteristics of total protein synthesis by isolated subcellular fractions. (a) Microsomal fraction. Separate variation of the concentration of ATP, phosphoenolpyruvate, cell sap, and Mg^{2+} ions in the incubation mixtures showed that the optimum conditions for the incorporation of amino acid into protein by the isolated microsome fraction were: microsome suspension 0.4 ml., cell sap 0.2 ml., ATP 2 μmoles , phosphoenolpyruvate 20 μmoles in a total volume of 1.0 ml. and at a final Mg^{2+} ion

concentration 6 mM. Addition of pyruvate kinase was without effect on the incorporation.

When cell sap was treated by gel filtration with Sephadex G-25, a threefold stimulation of incorporation was obtained over that with untreated cell sap. Under these conditions the incorporation was doubled when 0.25 μmole of GTP was added to each ml. of incubation mixture. It seems likely that the beneficial effect of treatment of the cell sap with Sephadex is due to the removal of free amino acids which otherwise decrease the specific radioactivity of the added ^{14}C -labelled amino acid.

The incorporation of [^{14}C]phenylalanine showed a nearly linear response to polyuridylic acid at a concentration of 25 and 50 μg . of polyuridylic acid/ml. This result indicates that the codon for phenylalanine is the same as that found for other tissues.

After incubation of the microsomal fraction for 40 min. with [^{14}C]leucine the protein in the soluble fraction had a specific radioactivity 25% that of the particulate protein.

Determination of the RNA/protein ratio of the microsome fraction gave values between 0.35 and 0.38. This is much higher than the ratio 0.2 usually

Fig. 1. Incorporation of [^{14}C]lysine into protein fractions during the incubation of slices of lactating guinea-pig mammary gland. Slices (10 g.) were incubated in a solution containing glucose under $\text{O}_2 + \text{CO}_2$ (95:5) at 37° with 50 μC of [^{14}C]lysine. At the appropriate time the slices were separated from the incubation medium, disintegrated, and the various subcellular fractions isolated. Casein and α -lactalbumin were isolated from the medium as described in the Materials and Methods section. $\circ$ — $\circ$, 800g particulate fraction; $\bullet$ — $\bullet$, mitochondrial fraction; $\square$ — $\square$, microsome fraction; $\triangle$ — $\triangle$, cell sap; $\triangle$ — $\triangle$, casein from incubation medium; $\circ$ — $\circ$, α -lactalbumin from the incubation medium.

Fig. 2. Separation of free and bound ribosomes of the microsome fraction from lactating guinea-pig mammary gland according to the method of Henshaw *et al.* (1963). Fraction 1 is from the bottom of the gradient. The peak around Fraction 7 contains the more dense 'bound' ribosomes and that around tube 32, the less-dense 'free' ribosomes.

obtained for the microsomal fraction from rat liver (Campbell, Cooper & Hicks, 1964) and suggested that the fraction might be rich in ribosomes which were not attached to membrane. This possibility was investigated by using the sucrose-density-gradient method of Henshaw *et al.* (1963). As shown in Fig. 2, a substantial peak of free ribosomes is detected in the microsome fraction from mammary gland. The proportion of free ribosomes is much greater than in the corresponding fraction from adult rat liver.

(b) Combined microsomal and mitochondrial fraction. The results presented in Table 1 show that the mitochondrial fraction is as active as the microsomal for cell-free protein synthesis, a result consistent with the findings in Fig. 1 and the report of Fraser & Gutfreund (1958).

For further experiments, the combined microsomal and mitochondrial fraction was used. Incorporation of amino acids into protein by this fraction was almost completely dependent on ATP and phosphoenolpyruvate, and also 50% dependent on exogenous GTP (Table 2). These characteristics are very similar to those of the microsome fraction previously described.

Biosynthesis of α -lactalbumin. To determine the nature of the protein being synthesized by the cell-free system from mammary gland use was made of an antiserum prepared against the whey proteins

Table 1. *Amino acid-incorporating activity of isolated microsomal and mitochondrial fractions from lactating guinea-pig mammary gland*

Each tube contained 0.4 ml. of washed particle suspension, 0.2 ml. of Sephadex-treated cell sap, 0.1 ml. of medium A, 2 μ moles of ATP, 20 μ moles of phosphoenolpyruvate, 0.25 μ mole of GTP and 0.25 μ c (0.67 μ g.) of [14 C]phenylalanine in total volume 1.0 ml. Radioactivity is expressed as counts/min. in protein plated at infinite thickness.

Fraction	Radioactivity of protein	Wt. of protein (mg.)
Mitochondrial	1403	5.5
Microsomal	1446	4.8

Table 2. *Requirements for amino acid incorporation by the combined microsomal and mitochondrial fractions from lactating guinea-pig mammary gland*

The complete system contained 0.4 ml. of washed particles, 0.3 ml. of Sephadex-treated cell sap, 3.6 μ moles of 2-mercaptoethanol, 2 μ moles of ATP, 20 μ moles of phosphoenolpyruvate (PEP) and 0.25 μ c of [14 C]leucine in a total volume 1.12 ml. Radioactivity is expressed as counts/min. at infinite thickness.

System	Radioactivity of protein
Complete	1480
Without GTP	730
Without ATP, PEP and GTP	48

soluble in 0.03 N-hydrochloric acid in 85% ethanol. This fraction contains only serum albumin and α -lactalbumin (Brew & Campbell, 1967) and since serum albumin is not synthesized by the guinea-pig gland (Turba & Hilpert, 1961c) such an antiserum can be used to study the synthesis of α -lactalbumin.

Following the method of Campbell *et al.* (1960), two fractions from the incubation mixture were examined, the soluble fraction and that fraction of the particles rendered soluble by treatment with ultrasonic vibrations. The non-specific radioactive material present in each fraction was removed by co-precipitation by the addition of ovalbumin and antiserum to ovalbumin. The radioactivity of the various fractions was determined and the results are given in Table 3. The results are consistent with an ATP-dependent synthesis of α -lactalbumin, the radioactive protein being retained by the particles but released by ultrasonic vibrations. The high activity of the ovalbumin precipitates from the soluble fraction compared with the activity of the lactalbumin precipitates indicates that there is a considerable amount of non-specific contamination in this fraction and no conclusions can be drawn.

Table 3. *Incorporation of [¹⁴C]leucine into specific whey protein by the isolated microsome fraction from guinea-pig mammary gland*

The complete system contained 0.4 ml. of microsome suspension, 0.2 ml. of cell sap, 0.1 ml. of medium A, 2 μ moles of ATP, 15 μ moles of phosphoenolpyruvate (PEP), 50 μ g. of pyruvate kinase and 1 μ C of [¹⁴C]leucine in a total volume 1.0 ml. Lactalbumin is the material precipitating with antibody to the ethanol-soluble whey proteins. Specific radioactivities are expressed as counts/min./mg. of total protein or counts/min./mg. of specific protein.

System	Incubation time (min.)	Ultrasonic microsome residue sp. activity	Ultrasonic microsome supernatant			Incubation supernatant		
			Ovalbumin	Lactalbumin		Ovalbumin	Lactalbumin	
				Total counts	Sp. activity		Total counts	Sp. activity
Complete	30	9460	75	236	2565	394	73	341
	90	5910	62	193	1286	471	95	352
Without ATP, PEP	90	345	14	14	113	11	5	29

Further studies demanded a method which would allow the radioactive α -lactalbumin to be isolated from the incubated particles. Since the combined microsomal mitochondrial fraction provided the most active system a large-scale incubation with [³H]leucine was set up. The incubation contained 16 ml. of suspended particles, 12 ml. of Sephadex-treated cell sap, 800 μ moles of phosphoenolpyruvate, 80 μ moles of ATP, 40 μ moles of GTP and 800 μ C of L-[4,5-³H₂]leucine (specific activity 8.3 c/m-mole) in a total volume 40 ml. After incubation for 40 min. the particles were recovered, treated with ultrasonic vibrations and α -lactalbumin was isolated from the supernatant after addition of carrier milk. The α -lactalbumin contained 845 000 disintegrations of ³H/min.

To determine the specific radioactivity of the [³H]leucine in the various leucine residues of the α -lactalbumin the double-labelling technique of Dintzis (1961) was employed. For this purpose a sample of α -lactalbumin uniformly labelled with [¹⁴C]leucine was required.

In a previous experiment (see the first section under Results above, and Fig. 1) slices of mammary gland were incubated with [¹⁴C]lysine for 30 and 90 min. The specific radioactivity of the *N*-terminal and internal lysine residues of the α -lactalbumin isolated in these experiments was determined. The ratio of the *N*-terminal to internal specific radioactivity was 0.96 at 30 min. and 1.09 at 90 min. These results indicate that both preparations were within the limits of the experiment uniformly labelled.

Since the α -lactalbumin obtained from the incubation of slices of mammary gland was uniformly labelled a preparation labelled with [¹⁴C]leucine was obtained as follows.

Slices (40 g.) were incubated with 60 μ C (336 μ g.) of [¹⁴C]leucine for 3.5 hr. The α -lactalbumin was

Fig. 3. Separation of the tryptic peptides of reduced carboxymethylated guinea-pig α -lactalbumin. The numbered peptides are those found to have radioactivity after incubation of tissue slices with [¹⁴C]leucine.

isolated by CM-cellulose chromatography followed by gel filtration on Sephadex G-100. The protein (3.3 mg.) had a specific radioactivity (gas-flow counter) 21 200 counts/min./mg.

Portions of the α -lactalbumins labelled in the cell-free system with [³H]leucine and in the slice system with [¹⁴C]leucine were mixed, the disulphide bridges split by reduction or oxidation and the preparation was digested with trypsin or chymotrypsin. The peptides were separated by the 'fingerprint' technique. The results with tryptic digestion are shown in Fig. 3 and with chymotrypsin in Fig. 4. The ³H/¹⁴C ratio of all peptides

Fig. 4. Separation of the chymotryptic peptides of per-formic acid-oxidized guinea-pig α -lactalbumin. The numbered peptides are those found to have radioactivity after incubation of tissue slices with $[^{14}\text{C}]$ leucine.

Fig. 5. Isotope ratios in the tryptic peptides of guinea-pig α -lactalbumin. ●, Protein (110000 disintegrations/min.) labelled by incubation with $[^3\text{H}]$ leucine for 40 min. in the cell-free system was mixed with protein (24500 disintegrations/min.) labelled with $[^{14}\text{C}]$ leucine by slices of mammary gland and 0.8 mg. of unlabelled carrier protein before reduction, carboxymethylation and digestion with trypsin. ○, ^3H -labelled protein (110000 disintegrations/min.) was digested with 1% (w/w) of carboxypeptidase A for 16 hr. before mixing with ^{14}C -labelled protein (15500 disintegrations/min.) and treating as described above. The peptides were separated by 'fingerprinting', and the peptide numbers are those given in Fig. 3.

Fig. 6. Isotope ratios in the chymotryptic peptides of guinea-pig α -lactalbumin. Protein (110000 disintegrations/min.) labelled by incubation with $[^3\text{H}]$ leucine for 40 min. was mixed with the ^{14}C -labelled protein (24500 disintegrations/min.) and 0.8 mg. of carrier protein. The material was oxidized with performic acid and digested with 1% (w/w) of chymotrypsin. ○, Ratios in those peptides containing significant counts. The numbering of the peptides is that given in Fig. 4.

detected by spraying with ninhydrin was determined. The results with trypsin digestion are shown in Fig. 5 and with chymotrypsin in Fig. 6. It will be observed that in both cases there was a considerable distribution in the $^3\text{H}/^{14}\text{C}$ ratios, indicating non-uniformity of labelling of the leucine residues.

Digestion of $[^3\text{H}]$ leucine-labelled protein with carboxypeptidase A before mixing with $[^{14}\text{C}]$ -leucine-labelled protein produced no relative change in the ratio of any of the tryptic peptides (Fig. 5).

In an attempt to determine the direction of assembly of the polypeptide chain of α -lactalbumin the mitochondrial-microsome fraction was incubated with $[^{14}\text{C}]$ lysine for 40 min. The conditions were 12 ml. of suspended particles, 9 ml. of Sephadex-treated cell sap, 600 μmoles of phosphoenolpyruvate, 60 μmoles of ATP, 30 μmoles of GTP and 50 μC of L- $[^{14}\text{C}]$ lysine (specific activity 136 mc/m-mole). From this incubation 4.46 mg. of α -lactalbumin containing 2268 counts/min./mg. (gas-flow counter) was isolated. The N-terminal lysine had a specific activity 445 counts/min./ μmole and the internal lysines 554 counts/min./ μmole , giving a ratio 0.80.

DISCUSSION

Three aspects of the present work require discussion. Firstly, the implications for the morphological site of synthesis of milk protein;

secondly, the extent to which the biosynthetic mechanism in lactating mammary gland is similar to that of other tissues; thirdly, the degree to which the subcellular system mimics the intact cells in its ability to synthesize a specific protein.

On the first point, the studies with tissue slices demonstrate that the fraction of the disintegrated cells with the sedimentation properties of mitochondria, the mitochondrial fraction, is the most active for protein synthesis in the intact mammary cell. Moreover, other experiments showed that the isolated mitochondrial fraction is at least as active as the microsomal fraction for protein synthesis under conditions that would be expected to be optimum for protein synthesis by cytoplasmic ribosomes. The results are entirely in agreement with those of Fraser & Gutfreund (1958) already referred to in the introduction. The most likely explanation for the relatively high activity of the mitochondrial fraction is that fragments of the rough-surfaced endoplasmic reticulum, which is a prolific component in cells of lactating mammary glands (Bargmann & Knoop, 1959; Hollmann, 1959), are distributed among the different subcellular fractions. The rapid sedimentation of such fragments in the mitochondrial fraction might be expected to deplete the microsome fraction and leave it relatively rich in ribosomes unattached to membrane; this was indeed found. The results from experiments on the liver-microsome fraction, which is rich in membranes, show that newly synthesized protein is retained by the membranes. If therefore the mitochondrial fraction from mammary gland contains such membranes it might have a higher concentration of newly synthesized protein than the microsome fraction, from which the synthesized protein would be released more rapidly. These observations would provide a satisfactory explanation for the results shown in Fig. 1, where the protein of the mitochondrial fraction had a higher specific radioactivity than that of the microsome fraction.

Since the present work was done with tissue homogenates prepared in 0.15M-sucrose it is perhaps not surprising that the fraction sedimenting at 11500g, which is usually referred to as the mitochondrial fraction, was very low in neotetrazolium succinate reductase, an enzyme confined to mitochondria (K. Brew, unpublished work). Peters (1957) has shown that in homogenates of rat liver prepared in 0.02M-sucrose most of the mitochondria sediment at 800g. The fact that the fraction from mammary gland sedimenting at 11500g contained virtually no mitochondria is not therefore so unexpected. It may be noted that with homogenates of hen's oviduct Hendler (1957) found that most of the fragments of the rough endoplasmic reticulum sedimented at 600g.

The studies of the biosynthesis of α -lactalbumin by slices of lactating mammary gland first confirm the finding of Turba & Hilpert (1961c) that this protein is synthesized on incubation of whole cells. The authors assumed that the protein was homologous with cow β -lactoglobulin, but were undoubtedly studying the protein now identified as α -lactalbumin. As with the synthesis of serum albumin by slices of liver (Peters, 1957; Campbell & Stone, 1957), a delay of about 15min. is seen before the labelled protein appears in the incubation medium (Fig. 1). Canfield & Anfinsen (1963) have demonstrated that complete lysozyme molecules are synthesized by minced hen's oviduct in less than 3min. α -Lactalbumin has virtually the same number of amino acid residues as lysozyme and may be synthesized in a similar time. Thus the present results with α -lactalbumin emphasize again that the time taken to export a protein from a cell may be much greater than the time taken for its synthesis.

The optimum conditions for the incorporation of ^{14}C -labelled amino acid by either the isolated microsome fraction or the combined mitochondrial and microsome fractions showed no marked differences from those found with the liver-microsome fraction. The ATP-dependence of incorporation into the protein precipitated by antibody to the ethanol-soluble whey proteins suggests that the requirements for α -lactalbumin synthesis are similar to those for total protein synthesis in the tissue. As with serum albumin synthesized by the microsomal fraction from rat liver (Campbell *et al.* 1960) the α -lactalbumin synthesized during incubation of the cell-free systems is apparently bound to the membranes of the rough endoplasmic reticulum being released by a combination of ultrasonic vibrations and freezing-and-thawing.

In experiments designed to demonstrate the synthesis of a specific protein in a cell-free system care must be taken to ensure that the radioactive protein that is isolated is properly characterized and that the apparent radioactivity is not due to contamination. When α -lactalbumin was purified from the incubation mixtures by rigorous methods involving ion-exchange chromatography and gel filtration it remained radioactive. Treatment of α -lactalbumin with trypsin gives the expected number of peptides based on the content of arginine and lysine. When the α -lactalbumin containing $[^3\text{H}]$ leucine from the cell-free system was mixed with $[^{14}\text{C}]$ leucine α -lactalbumin from the tissue-slice incubation, $[^3\text{H}]$ leucine was only found in peptides that also contained $[^{14}\text{C}]$ leucine, even though all significant peptides that reacted with ninhydrin on the 'fingerprint' were examined. Similar results were obtained in identical experiments in which chymotrypsin was used for hydro-

lysis instead of trypsin. This is good evidence that the [^3H]leucine associated with the α -lactalbumin from the cell-free system was in fact incorporated into the polypeptide chain of α -lactalbumin.

The lack of uniformity in the specific radioactivity of the leucine residues in the peptides isolated from the α -lactalbumin, labelled during incubation of the combined mitochondrial and microsome fraction, suggests the completion of polypeptide chains from peptide precursors attached to the particles. Many other proteins have been shown to be synthesized by a stepwise assembly of amino acid residues and in all these the direction of assembly has been from the *N*-terminus. The operation of a similar mechanism for α -lactalbumin is supported by the lower specific radioactivity of the *N*-terminal lysine as compared with the other lysine residues when the subcellular fraction was incubated with [^{14}C]lysine for 40 min. The specific activity of the *N*-terminal lysine was, however, 80% that of the other residues. This result is at first sight difficult to reconcile with the wide range of isotope ratios in the leucine-containing peptides. All but two of the tryptic peptides would have contained lysine, the other two containing arginine. It can be shown (K. Brew, unpublished work) that the two peptides containing the most active leucine were positive to the Sakaguchi reaction, i.e. they contained arginine and not lysine. Thus the lysine-containing peptides may be concentrated towards the *N*-terminus of the protein. The fact that the *N*-terminal lysine was so actively labelled indicates that new polypeptide chains are readily initiated in this cell-free system from mammary gland.

The distribution in the specific radioactivity of the leucine residues of α -lactalbumin does not conform to a smooth curve (see Figs. 5 and 6) but rather suggests that the residues fall into two groups. This suggests that the polypeptide chain of α -lactalbumin is not assembled at a uniform rate throughout its length. Various reasons may be advanced for this, the most likely being, bunching of ribosomes towards one end of the messenger RNA for α -lactalbumin, certain species of transfer RNA are rare and so the addition of one particular amino acid is rate-limiting, the formation of peptide bonds between certain amino acids is slower than for the rest. It is not possible to choose at this stage between these different possibilities.

α -Lactalbumin is rich in disulphide bonds having eight half-cystine residues per molecule (Brew & Campbell, 1967). From the behaviour of the radioactive protein isolated from the cell-free system on ion-exchange chromatography and gel filtration it may be assumed that the newly synthesized protein is in a native state. It follows that the correct secondary and tertiary structure of the protein must be achieved in the cell-free system and

in particular that the 'correct' disulphide bonds are forged. Possibly an enzyme catalysing these processes is present in mammary gland as has been shown for rat liver (Goldberger, Epstein & Anfinsen, 1963).

These experiments in which it is demonstrated that a specific milk protein is synthesized by a relatively simple biological system open up the possibility of a new approach to the problem of the initiation and control of synthesis of milk protein. That this process can be studied in organ cultures of mammary tissue has already been demonstrated by the hormonal induction of synthesis of milk protein in such an isolated preparation (Juergens, Stockdale, Topper & Elias, 1965; Turkington, Juergens & Topper, 1965).

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