CHANGES IN THE RIBOSOME CONTENT, PRINCIPAL MICROSOMAL PROTEIN COMPOSITION, AND SECRETORY CHARACTER OF MAMMARY EPITHELIAL ROUGH ENDOPLASMIC RETICULUM DURING DIFFERENTIATION

Evidence that Messenger RNAs Specific for Milk Proteins are Incorporated into Rough Endoplasmic Reticulum Formed *De Novo* after Parturition

FRANK SLABY and CAROLYN BROWN

From the Department of Biology, Joseph Leidy Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania 19174 and the Worcester Foundation for Experimental Medicine, Shrewsbury, Massachusetts 01545. Dr. Brown's present address is Mount Holyoke College, South Hadley, Massachusetts 01075.

ABSTRACT

The equilibrium density distribution, protein composition, and secretory character of mouse mammary epithelial rough microsomes have been determined during differentiation. The density range exhibited by the rough microsomes broadens during mammary development; rough microsomes within the 1.25–1.29 g/ml density range appear soon after conception and then within the 1.30–1.34 range after the onset of lactation. The appearance of these denser microsomes represents the progressive increase of the average ribosome content of the rough endoplasmic reticulum (ER) during gestation and lactation. Fractionation of rough microsomal proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals that two proteins, having molecular weights of 57,000 and 76,000, occur to a significant extent only during lactation and are then most prominent in the very dense rough microsomes of the 1.30–1.34 range. Nascent polypeptide chains discharged (by incubation with puromycin) from 17-days lactation rough microsomes in either the 1.21–1.29 or 1.30–1.34 density range are distributed equally between the intra- and extravesicular compartments. Whereas 36% of the chains are discharged intravesicularly from 1-day lactation rough microsomes in the 1.30–1.34 range, only 25% are so discharged from those in the 1.21–1.29 range. The results indicate (*a*) that there is no correlation be-

tween the relative levels in lactation rough microsomes of the two microsomal proteins which become prominent during lactation and the extent of secretory activity and (*b*) that for a short period after parturition the rough ER elements bearing high surface densities of ribosomes have a greater proportion of ribosomes synthesizing milk proteins than the rough ER elements with moderate ribosome densities.

INTRODUCTION

The mammary gland alveolar epithelium of a nulliparous midpregnant mouse consists predominantly of unspecialized, nonsecretory cells which characteristically have an extremely sparse content of rough endoplasmic reticulum (ER)[1] (1). By contrast, these cells 9–10 days later, at the time of parturition, exhibit a pronounced ultrastructural specialization characterized by a well-developed rough ER system and the presence of secretory products (2). After the onset of lactation there is a further accumulation of rough ER which coincides with its elaboration into a pattern similar to that observed in pancreatic alveolar epithelium (3).

Oka and Topper (4) have shown from in vitro studies that the formation of an extensive rough ER system precedes initiation of casein synthesis. Since the membrane-bound ribosomes account for most of the milk protein synthesis (5), it is evident that there is a substantive change in the kinds of proteins synthesized and the manner in which they are vectorially discharged by the rough ER near the time of parturition. We have been interested in whether acquisition of this capacity to synthesize and export milk proteins is accompanied by changes in the major membrane protein complement of the rough ER. The rough ER system, however, cannot be isolated as an intact subcellular structure; the shear forces generated during homogenization render most of the system into microsomes, closed, approximately spherical, vesicles ranging 100–200 nm in diameter (6, 7). Centrifugation of the homogenate at 10,000 *g* for 10–20 min sediments the nuclei, mitochondria, and a significant amount of material derived from the rough ER system (8). The vesiculated rough ER elements in suspension can be sedimented by centrifugation at 100,000 *g* for 60 min. Although this microsome fraction is dominated by vesicles derived from smooth and rough ER, it also contains free ribosomes and vesicles of plasma membrane, outer nuclear and mitochondrial membrane, Golgi apparatus, and lysosome origin. The sharp segregation of function among these membrane systems in the intact cell can be retrieved in the microsome fraction only to the extent to which the functional distinctions are manifested in physiochemical properties (such as size, density, or charge) of the vesiculated fragments. We have applied isopycnic density gradient centrifugation in linear sucrose gradients of low ionic strength, conditions under which the equilibrium density of a microsomal vesicle is a correlate of both its RNA and phospholipid (PLP) content (9). In practice, the vesicles are separated into basically two classes, one being predominantly derived from rough ER (with its high RNA content) and the other from smooth ER, Golgi apparatus, and plasma membrane (with their low RNA and/or high PLP content) (10, 11).

Our analysis of the rough vesicles in microsomes isolated from mouse mammary epithelium during gestation and lactation indicates that a progressive increase of the ribosome content and changes in the major protein complement of the rough ER attend its accumulation during these periods. The most heavily ribosome-laden vesicles are encountered during lactation and they exhibit to a greater degree the changes in major microsomal protein composition which occur upon parturition than do rough vesicles of lesser ribosome content. The very heavily ribosome-laden rough vesicles have greater secretory activity than the vesicles of lesser ribosome content at 1-day lactation but not at 17-days lactation. The results indicate (*a*) that, immediately after parturition, messenger RNAs specific for milk proteins are incorporated into rough ER elements formed independently of preexisting rough ER structures and (*b*) that the changes in major microsomal

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; PLP, phospholipid; SDS, sodium dodecyl sulfate.

protein composition which occur after parturition do not account for the acquisition of secretory activity by the rough ER.

MATERIALS AND METHODS

Isolation of Epithelial Cells

Mammary tissue consists principally of a mixed population of epithelial and adipose cells with vascular, neural, and connective tissue elements (12). After dispersion of the tissue with collagenase, the dissociated epithelial and adipose cells can be separated by taking advantage of the difference in their density (13); cell preparations containing primarily epithelial cells can thus be obtained (14, 15). Virginal, nulliparous pregnant, and primiparous lactating and nonlactating mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) of the CD-1 strain were killed by cervical dislocation. The abdominal and thoracic mammary glands were immediately placed in Medium 199 with Hanks' salt solution (Microbiological Associates, Inc., Bethesda, Md.) upon excision and minced with scissors. Crude collagenase (Worthington Biochemical Corp., Freehold, N. J.) was added to a concentration of 0.8 mg/ml and the suspension incubated at 37°C for 90 min in a water bath with shaker. The tissue minces were repeatedly passed through a 10-ml plastic pipette at 20-min intervals during the incubation to facilitate tissue dispersion. Upon removal of intact tissue fragments and large connective tissue strands by passage through a fiber glass screen, the dissociated epithelial cells were separated from fat cells by centrifugation at 200 *g* for 5 min at 20°C. The cell pellet was washed three times with 20 vol of Medium 199.

Preparation of Microsomes

Epithelial cell preparations were homogenized in 5–10 vol 0.25 M sucrose, 5 mM $MgCl_2$, and 10 mM Tris (pH 7.6) with 10 strokes of a Teflon homogenizer (0.004–0.006 inch clearance). In order to both reduce the amount of ER material discarded with the nuclear-mitochondrial pellet and yet minimize nuclear disruption by excessive homogenization, the homogenate was centrifuged at 500 *g* for 5 min and the supernate homogenized further (15 strokes) before being centrifuged at 10,000 g_{av} for 15 min to remove any residual nuclei and mitochondria. The supernate was then centrifuged at 200,000 g_{av} for 60 min. The microsomal pellet was suspended and homogenized in 0.4 ml 0.25 M sucrose and 10 mM Tris (pH 7.6) and layered over a 4.6 ml linear 1.0–2.0 M sucrose gradient (density 1.15–1.34 g/ml) in 10 mM Tris (pH 7.6). Microsome subfractionation by density equilibration was achieved by centrifugation at 230,000 g_{av} for 11 h in Beckman SW-50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 12 equal fractions were collected with an LKB Varioperpex peristaltic pump[2] (LKB Instruments, Inc., Rockville, Md.). Pelleted material was suspended in 0.5 ml 20 mM Tris (pH 7.6).

Determination of Free Ribosome Content of Microsome Preparations

The proportion of ribosomes in a microsome preparation which are membrane free was determined according to a modification of the method of Webb et al. (16). Two equal volumes of a microsome suspension were each diluted to 1.5 ml 1.0 M sucrose, 5 mM $MgCl_2$, and 10 mM Tris (pH 7.6); one suspension contained additionally 1% sodium deoxycholate. Both suspensions were layered over 1.5 ml 2.0 M sucorse, 5 mM $MgCl_2$, and 10 mM Tris (pH 7.6), overlaid with 2.0 ml 0.9 M sucrose, 5 mM $MgCl_2$, and 10 mM Tris (pH 7.6), and centrifuged at 100,000 g_{av} for 20 h in a Beckman SW-39L rotor. Ribosomal pellets were suspended in 1.0 ml 20 mM Tris (pH 7.6).

Biochemical and Enzymatic Assays

Protein was determined according to the method of Lowry et al. (17) using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard. Allowance for the interference of sucrose in this assay (18) was made by adjusting the protein determinations on 100-μl aliquots of the gradient fractions according to the effects 100 μl of 1.0, 1.5, and 2.0 M sucrose had in each assay on the measurement of bovine serum albumin over the 20–80 μg range.

RNA was determined by absorbance at 260 nm of microsome suspensions upon clarification in 1% sodium dodecyl sulfate (SDS), 0.005 M EDTA (sodium salt), and 0.1 M Tris (pH 7.0). After allowance was made for the contribution of protein to the OD_{260} (an absorbance of 0.066 for 100 μg protein/ml), the amount of RNA was calculated on the basis of an OD_{260} of 0.0275 for 1.0 μg RNA/ml.

Phospholipid was extracted from microsome suspensions with 20 vol of chloroform-methanol 2:1 (vol/vol). PLP phosphorus was determined according to Bartlett (19). The amount of phosphorus was multiplied by 25 to give the corresponding amount of PLP.

NADH- and succinate-cytochrome *c* reductase activities were determined spectrophotometrically by the oxidation of reduced cytochrome *c* (20, 21). NADH-cytochrome *c* reductase activity was used to monitor recovery of ER material in the microsome fraction.

[2] The gradients were fractionated in such a manner that the last fraction collected (gradient fraction 12) contained material at the top of the gradient. The microsomal material recovered in particular gradient fractions is referred to as microsome density subfractions.

Succinate-cytochrome *c* reductase activity, which is associated with the inner membrane system of the mitochondrion, was used to estimate the extent of mitochondrial contamination in the microsome fraction.

Electrophoretic Fractionation of Microsomal Proteins

Microsomal proteins were fractionated by discontinuous SDS-polyacrylamide gel electrophoresis according to the technique of Neville (22), with the following specifications: The upper gel was 2.6% (wt/vol) acrylamide-0.0845% *N*,*N*′-methylenebisacrylamide; the lower gel 8% acrylamide-0.26% *N*,*N*′-methylenebisacrylamide. The lower gel buffer was 0.0308 N HCl-0.4244 Tris (pH 9.18). Gels were cast in 8-mm OD glass tubes, with the upper and lower gels being 2 and 9 cm, respectively, in length. This electrophoretic method separates polypeptide chains according to molecular weight only (23, 24) and provides reproducible high resolution electrophoretograms of membrane proteins in which molecular weights can be established with 5% confidence limits (22).

Samples were prepared for electrophoresis by mixing 4 vol of sample with 1 vol 5% SDS, 0.2 M dithioerythreitol, and 0.01% bromphenol blue in five times upper gel buffer. Samples were then placed in a boiling water bath for 1 min, cooled to room temperature, and applied directly on the gels. Proteins used as molecular weight standards were β-galactosidase (mol wt 130,000), phosphorylase a (mol wt 94,000), bovine serum albumin (mol wt 66,500), and ovalbumin (mol wt 43,000).

Gel densitometry was conducted with a Gilford spectrophotometer and model 2410 linear transport accessory (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), scanning at 550 nm for Coomassie blue. The region between the upper surface of the gel and component A served to establish the base line for the scans.

Preparation of Ribosomal Subunits from Lactation Rough Vesicles

Rough vesicles from 17-days lactation microsomes (those vesicles which are dispersed in the lower half of a linear 1.0–2.0 M sucrose gradient upon equilibrium density centrifugation) were suspended in 1% sodium deoxycholate, 0.5% Triton X-100, 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris (pH 7.6), layered over 1.0 ml 2.0 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris (pH 7.6), and centrifuged at 100,000 g_{av} for 18 h in an SW50L rotor. The ribosomal pellet was suspended in 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris (pH 7.6) and centrifuged at 8,000 g_{av} for 20 min to sediment aggregated material. Ribosomal subunits were prepared according to a modification of the method of Blobel (25). Ribosome preparations were incubated in 1.0 ml 500 mM KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM puromycin, and 50 mM Tris (pH 7.6) at 37°C for 20 min, layered on a 27.0 ml linear 5–20% sucrose gradient in 500 mM KCl, 2 mM $MgCl_2$, and 50 mM Tris (pH 7.6), and centrifuged at 21,000 rpm for 7 h in an SW25.1 rotor at 20°C. Ribosomal subunits were recovered from appropriate gradient fractions by centrifugation at 100,000 g_{av} for 24 h in an SW50L rotor at 4°C.

Vectorial Discharge of Nascent Polypeptide Chains by Puromycin

For amino acid incorporation in vitro, incubation tubes contained in a final volume of 1.0 ml: 50 μmol Tris-HCl (pH 7.5 at 25°C), 25 μmol KCl, 5 μmol $MgCl_2$, 1 μmol ATP, 0.4 μmol GTP, 10 μmol phosphoenolpyruvate, 10 μg pyruvate kinase, 2 μmol dithiothreitol, 0.1 μmol each of 19 L-amino acids, 5–10 μCi [^{3}H]leucine, and 0.1 ml pH 5 fraction (26). The reactions were started by adding rough vesicle suspensions (containing 100 μg RNA) to tubes prewarmed to 37°C and containing all the other components. Duplicate tubes were incubated at 37°C for 7 min and puromycin added to a final concentration of 1 mM. Control tubes received an equal volume of buffer (25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris [pH 7.5]). All tubes were then incubated an additional 12 min. Incubation was stopped by the addition of ice-cold buffer and the mixture centrifuged at 100,000 g_{av} for 90 min. The resulting supernate, considered "extravesicular," was precipitated with 5% TCA, while the resulting pellet was resuspended in 1.0 ml buffer containing 0.5% sodium deoxycholate, layered over 1.0 ml buffer containing 0.25 M sucrose, overlaid with buffer, and centrifuged at 100,000 g_{av} for 90 min. The pellet resulting from this centrifugation was considered "ribosomal" and the supernate considered "deoxycholate-released intravesicular" material. After resuspension of the ribosomal pellet, both the suspension and supernate were precipitated with ice-cold 5% TCA. Precipitates were washed twice in 5% TCA, incubated for 20 min at 80°C in 5% TCA, and extracted twice in ethanol:ether (3:1, vol/vol)before determining the radioactivity.

RESULTS

Equilibrium Density Dispersion of Microsomal Vesicles

Changes in the relative proportions of protein, RNA, and PLP in the microsome fraction of mammary epithelium during differentiation and regression are shown in Table I. There is a progressive accumulation of RNA during gestation and lactation, attaining a level in the 17-days lactation microsomes approximately six times that in the virgin microsomes; the RNA content de-

TABLE I
RNA and PLP Content of Mammary Epithelial Microsomes during Mammary Development

Stage of development	RNA to membrane protein mass ratio	PLP to membrane protein mass ratio
Virginity	0.04	0.54
12-days gestation	0.07	0.55
19-days gestation	0.14	0.52
2-days lactation	0.17	0.49
17-days lactation	0.23	0.49
5-days postlactation	0.05	0.51

Total protein, RNA, and PLP content in an aliquot of a microsome suspension were determined as described in Materials and Methods. The content of membrane protein was obtained by subtracting 80% of the RNA content (representative of the amount of ribosomal protein) from the total protein. The data representative of each stage of mammary development are the average of four determinations.

clines to the virgin level by 5 days after weaning. The PLP content relative to that of protein remains rather constant, declining somewhat during lactation. Approximately 6–9% of the mitochondria are recovered in these microsome preparations, as indicated by succinate-cytochrome *c* reductase activity. Assaying for NADH-cytochrome *c* reductase activity indicates that roughly half of the ER material is discarded with the nuclear-mitochondrial fraction.

The isopycnic density distribution of these microsomal constituents exhibits a progressive transformation as the mouse passes from virginity through pregnancy and the postpartum period (Fig. 1). The distribution pattern of microsomal protein in the virgin condition is characterized by a rather low modal equilibrium density (1.22) and the virtual absence of any protein at a density greater than 1.27. The distribution pattern of microsomal RNA is almost identical to that of protein. The distribution pattern of PLP exhibits a bimodal aspect; the denser mode is due to the predominance of microsomal material in the 1.20–1.25 density range, whereas the lighter mode is accounted for by the increased content of PLP relative to protein in the lighter microsome subfractions.

Soon after conception, the protein assumes a bimodal density distribution. The equilibrium density of the lighter mode remains constant (1.18) during gestation; that of the heavier mode, however, increases from 1.23 at midpregnancy (10–12 days) to 1.26 just before parturition (19–20 days). The RNA retains a unimodal distribution pattern whose equilibrium density occurs within the 1.25–1.27 density range during gestation. The proportion of microsomal material equilibrating within the 1.25–1.29 density range increases during the last week of gestation; the protein, RNA, and PLP contents increase by 80, 28, and 57%, respectively. Throughout gestation there are still no microsomal constituents exhibiting a density greater than 1.30.

The onset of lactation marks the initial appearance of microsomal material in the 1.30–1.34 density range. The microsomal material in the 1.30–1.34 density range accumulates during the postpartum period to the extent of becoming the predominant density class, accounting for one-third of the protein, one-half of the RNA, and one-quarter of the PLP. Although microsomal constituents now extend throughout the sucrose gradient, less than 1% of the protein or RNA layered on the gradient can be recovered as pelleted material. This figure also applies to the epithelial microsome preparations of virgin and pregnant mice.

The density distribution of the microsomal constituents 5 days after weaning is remarkably similar to that characteristic of the virgin condition. Although the protein exhibits a bimodal distribution pattern, the equilibrium density of the heavier mode is 1.23. There is also practically no microsomal material with a density greater than 1.27.

To explore the possibility that there is selective sedimentation of denser microsomes during the 10,000 *g*-15-min centrifugation of the homogenate, experiments were done in which homogenates of epithelial cell preparations from 19-days pregnant and 17-days lactating mice were centrifuged at 500 *g* and 10,000 *g* for 15 min and the density distribution of the material remaining suspended after each centrifugation then determined. Decreasing the centrifugation force substantially increases the amount of microsomal material recovered from the lactation homogenate (62% more protein and 52% more RNA), but only slightly increases the amount recovered from the late gestation homogenate (16% more protein and 11% more RNA). For both types of microsome preparations there are no qualitative differences between the distribu-

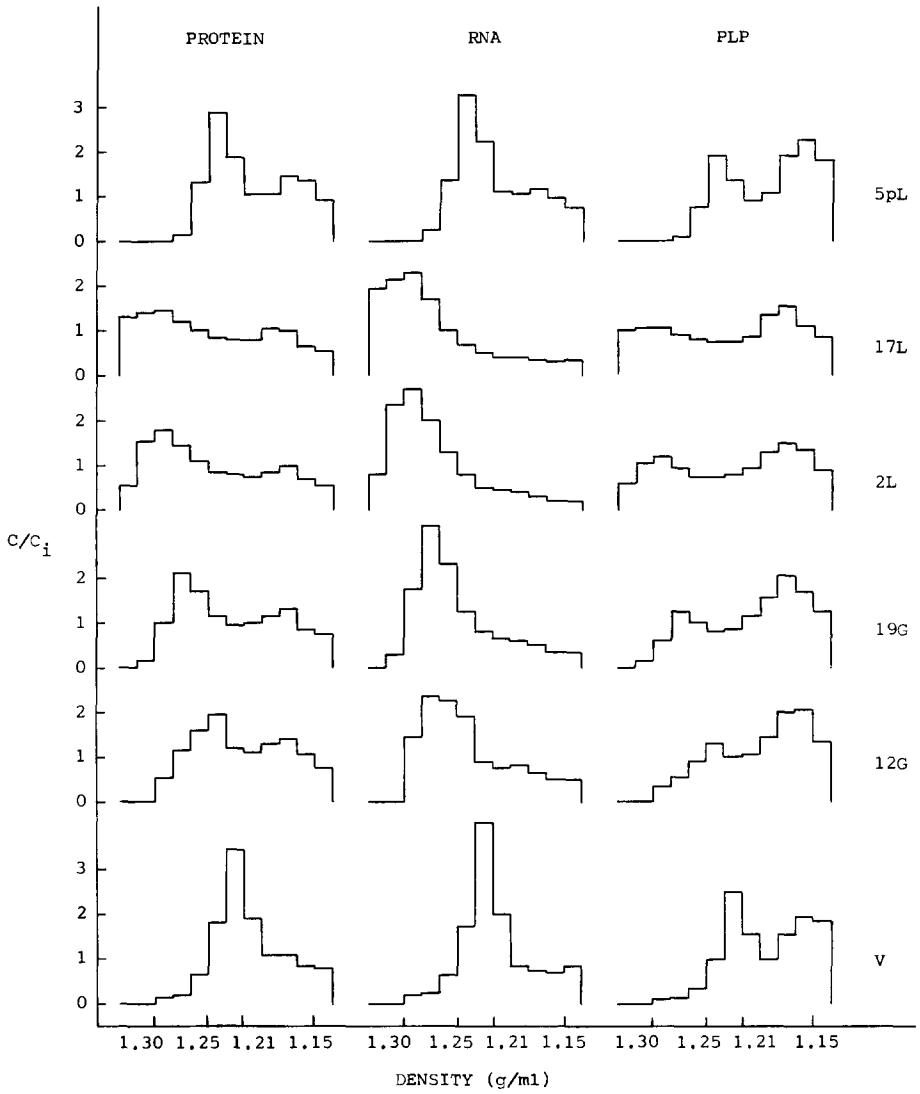

FIGURE 1 Isopycnic density distribution of microsomal protein, RNA, and PLP characteristic of virginity (V), 12-days gestation (12G), 19-days gestation (19G), 2-days lactation (2L), 17-days lactation (17L), and 5-days postlactation (5pL). C/C_i is the relative concentration, that is, the ratio of the concentration C in the microsome subfraction to the concentration C_i that the constituent would have if it were uniformly distributed throughout the entire gradient. The right-hand edge of each histogram represents the relative concentration of the microsomal constituent at the top of the gradient. The distribution patterns representative of each stage of mammary differentiation are the average of two or three experiments.

tion patterns of the 500 *g* and 10,000 *g* suspensions (Fig. 2). In the case of the 19-days gestation microsomes, decreasing the centrifugation force increases the proportion of microsomal material within the upper half of the gradient; it does not, however, result in the appearance of substantial microsomal material with a density greater than 1.30. The results show that decreasing the centrifugation force may slightly affect the proportion of microsomal material within certain density ranges but does not affect the density range exhibited by the microsome preparation.

Since the ribosome load of a vesicle contributes significantly to the vesicle's equilibrium density,

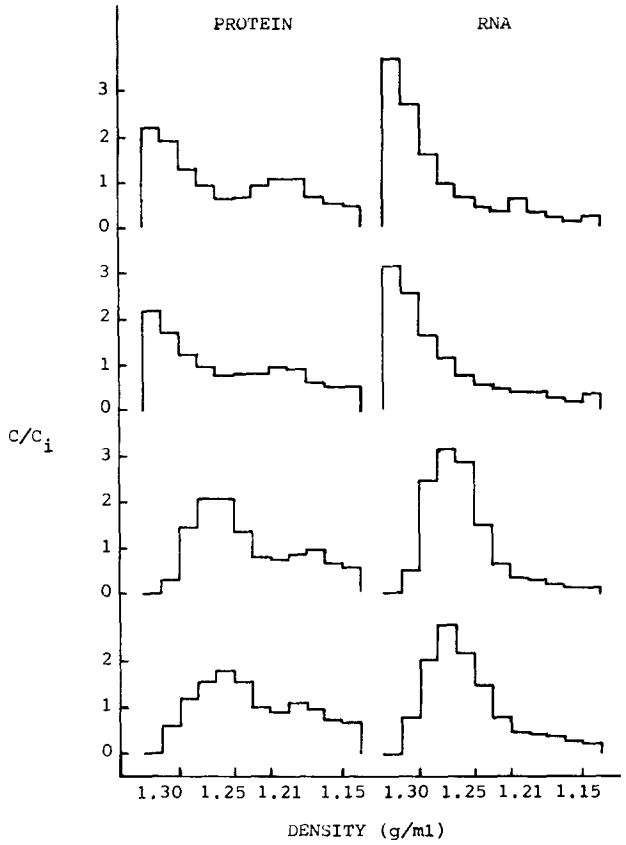

FIGURE 2 Density distribution patterns of microsomal constituents isolated at 17-days lactation and 19-days gestation. Top row: 17-days lactation microsomes were isolated and dispersed as discussed in Materials and Methods. Second row: The experimental conditions were the same as those pertaining to the top row except that the microsomes were isolated from a 15 min-500 g_{av} supernate of the homogenate. Third row: 19-days gestation microsomes were isolated and dispersed as discussed in Materials and Methods. Bottom row: The experimental conditions were the same as those pertaining to the third row except that the microsomes were isolated from a 15 min-500 g_{av} supernate of the homogenate.

we would expect that the vesicles in the gestation and lactation microsome preparations which equilibrate within the 1.27–1.34 density range have ribosome contents greater than that of vesicles of lesser density. To examine this possibility the mass ratio of RNA to microsomal protein, exclusive of ribosomal protein, was determined for the microsome subfractions within each gradient, assuming that the amount of ribosomal protein is equal to 80% of that of RNA (27). Such calculations reveal that the RNA to membrane protein mass ratio continuously increases with density for microsomes isolated during lactation or the last half of gestation (Fig. 3). By contrast, the mass ratio is almost constant throughout the gradients containing microsomes representative of the virgin, early gestation, or early weaning conditions. The RNA content of microsome subfractions of equivalent density is not the same in each gradient, but instead progressively increases during gestation, attaining a maximal level soon after parturition. The most extreme disparity is observed in the 10-fold difference between microsome subfractions 3 in the virgin and 17-days lactation gradients. The densest subfraction in the virgin gradient has, moreover, an RNA content less than that of the four lightest subfractions in five of the other gradients.

Similar calculations of PLP to membrane protein mass ratios show that in all gradients the ratio increases as one passes to successively lighter densities (Fig. 4). The PLP content of microsomal ma-

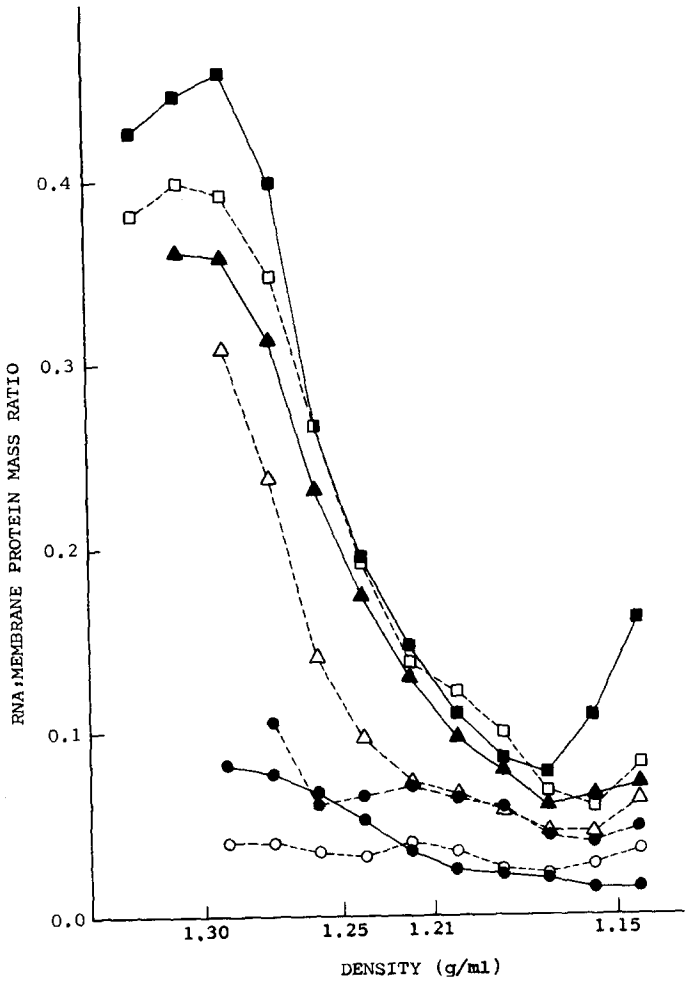

FIGURE 3 RNA to membrane protein mass ratio as a function of the density of the microsome subfraction in virgin (O- - -O), 7-days gestation (●——●), 12-days gestation (Δ- - -Δ), 19-days gestation (▲——▲), 2-days lactation (□- - -□), 17-days lactation (■——■), and 5-days postlactation (●- - -●) gradients. Except for the 7-days gestation curve, this family of RNA content curves has been determined from the data shown in Fig. 1.

terial with a density greater than 1.23 is seen to be within a limited range (0.3–0.5). The gross PLP-protein composition of the membranous compartment of these microsome subfractions thus appears to be roughly constant.

The extension of the microsome density range soon after conception and then again at the onset of lactation is thus correlated, in both instances, with the appearance of vesicles having greater RNA contents. The large increases in the RNA to membrane protein mass ratios encountered within equivalent density ranges during mammary development could be the result of a sedimentation pattern of free ribosomes superimposed upon an equilibrium pattern of ribosome-bearing vesicles. This might account for the absence of ribosome pellets. A test for such a superimposition was attempted through the following approaches:

(*a*) 17-days lactation microsomes were suspended in 2.0 M sucrose, layered under a linear 1.0–2.0 M sucrose gradient, and centrifuged at 230,000 g_{av} for 25 h. The density distribution patterns of protein and RNA were compared with those of the same microsome preparation suspended in 0.25 M sucrose and layered over the gradient (Fig. 5). The equilibrium density distribu-

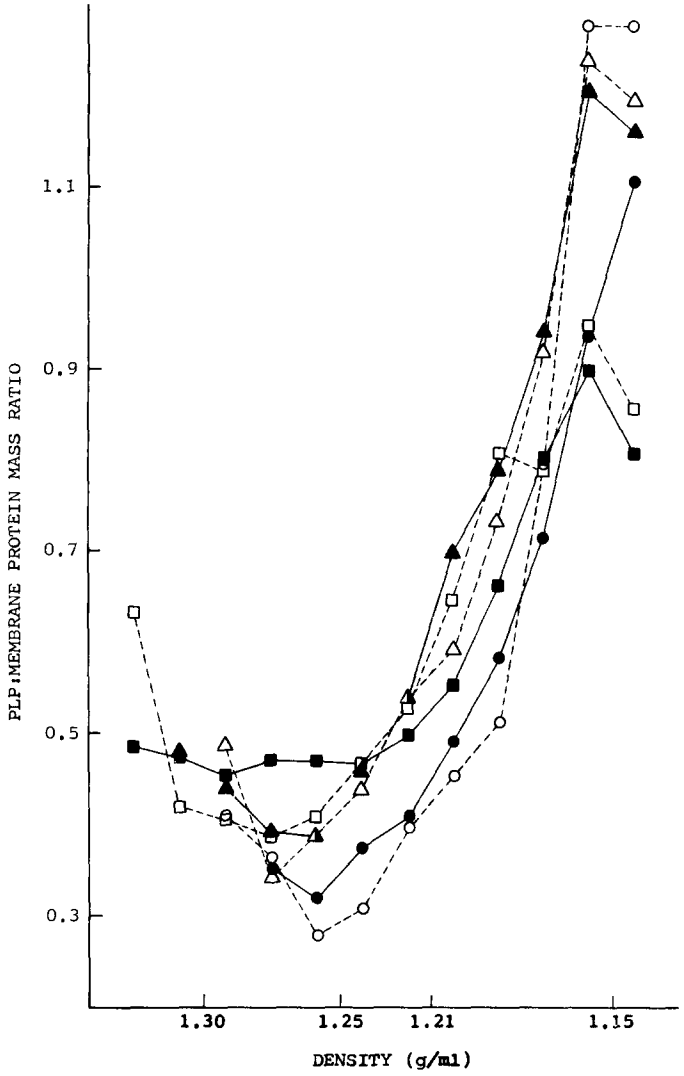

FIGURE 4 PLP to membrane protein mass ratio as a function of the density of the microsome subfraction in virgin (O- - -O),12-days gestation (Δ- - -Δ), 19-days gestation (▲——▲), 2-days lactation (□- - -□), 17-days lactation (■——■), and 5-days postlactation (●——●) gradients. This family of PLP content curves has been determined from the data shown in Fig. 1.

tion of lactation microsomes layered over the gradient and centrifuged for an extended period of 25 h is indistinguishable from that commonly obtained after 11-h centrifugation. 98% more protein and 72% more RNA are recovered at densities greater than 1.30 g/ml by layering the microsomes suspension under the gradient; there is a complementary smaller proportion of microsomal material in the upper half of the gradient. This difference in the microsome distribution patterns is probably due to a situation in which microsomal vesicles more readily migrate to their buoyant density positions if they have to migrate in the direction of the centrifugal force field rather than opposite to it. In both cases, less than 5% of the RNA applied to the gradient is recovered as pelleted material. These results indicate that measures which maximize the complete sedimentation of free ribosomes either through the gradient or from an underlaid microsome suspension still do

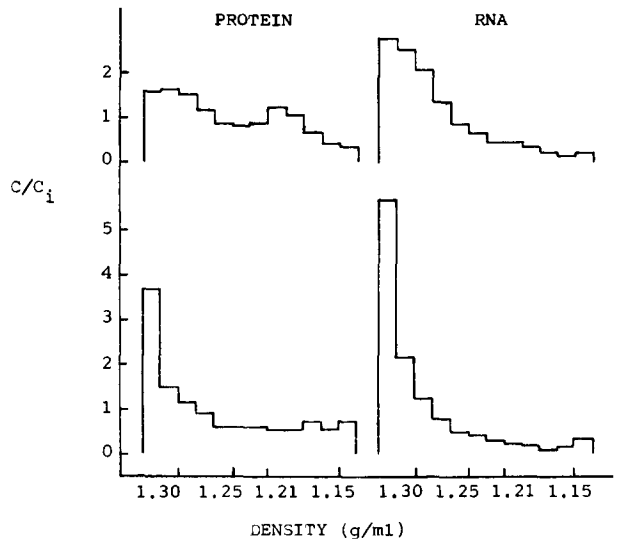

FIGURE 5 Density distribution patterns of microsomal constituents isolated at 17-days lactation. Top row: The microsomes were isolated and dispersed as discussed in Materials and Methods except that the duration of the sucrose gradient centrifugation at 230,000 g_{av} was 25 h. Bottom row: The same microsome preparation was suspended in 0.4 ml 2.0 M sucrose and 10 mM Tris (pH 7.6), layered under a 4.6 ml linear 1.0-2.0 M sucrose gradient in 10 mM Tris (pH 7.6), and centrifuged at 230,000 g_{av} for 25 h.

not yield more than a small percentage of the RNA as pelleted material.

(*b*) Sodium deoxycholate is a relatively mild anionic detergent which, at 1% concentrations, renders membrane-bound ribosomes virtually PLP free (8). If two equal portions of a microsome preparation, one of which has been treated with sodium deoxycholate, are layered over 2.0 M sucrose and centrifuged for a period sufficient to sediment membrane-free ribosomes, the amount of ribosomes pelleted from the untreated microsome suspension relative to the amount sedimenting after detergent treatment provides an indirect estimate of the proportion of free ribosomes in the microsome preparation (16, 28). Such an analysis, when applied to 19-days gestation and 17-days lactation microsomes, indicates that 6–12% of the ribosomes are membrane free.

These findings demonstrate that no more than 15% of the RNA in the gradients can be attributed to sedimenting membrane-free ribosomes. The density distribution patterns therefore represent the equilibrium distributions of the different kinds of vesicles in the microsome preparations.

SDS-Polyacrylamide Gel Electrophoresis of Microsomal Proteins

SDS-polyacrylamide gel electrophoresis resolves nine principal protein bands within the 30,000–200,000 molecular weight range from mouse mammary epithelial microsomes. The estimated molecular weights of these polypeptide components and the letters by which they are designated in this article are shown in Table II. The relative contents of all nine proteins in the total microsome fraction change significantly during mammary development and regression (Fig. 6). If the general levels of each component prior and subsequent to parturition are compared, three patterns emerge: (*a*) Components E and G are the only two proteins which appear as principal components at all stages of epithelial differentiation. At every stage component G is the most or second

TABLE II
Principal Proteins of Mammary Epithelial Microsomes

Polypeptide component	Molecular weight
A	200,000
B	155,000
C	98,000
D	76,000
E	68,000
F	57,000
G	42,000
H	35,000
I	33,000

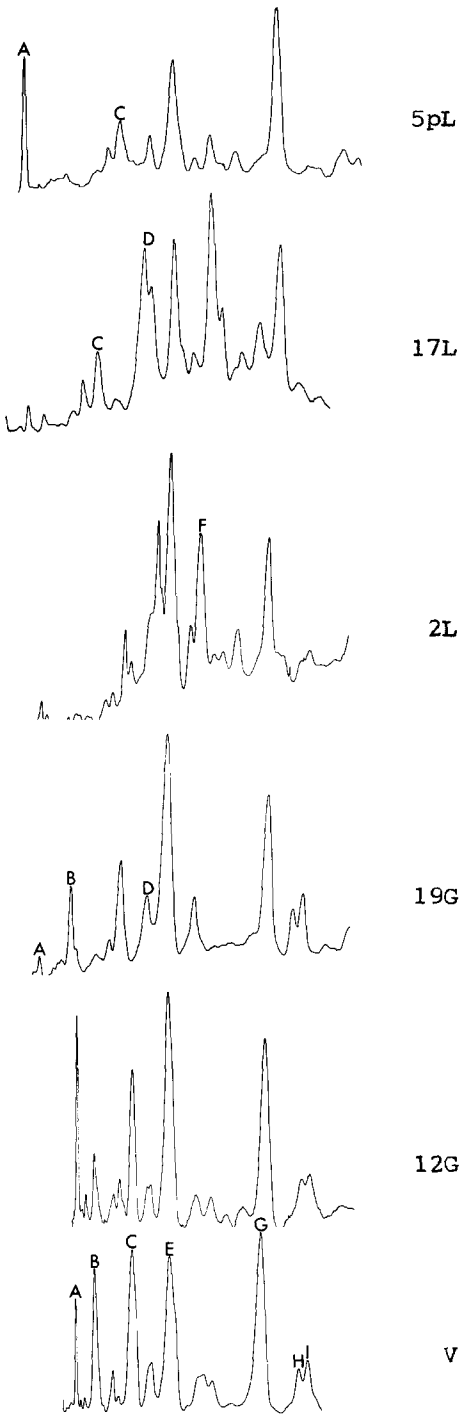

FIGURE 6 Densitometric tracings of electrophoretograms of microsome fractions representative of virginity (V), 12-days gestation (12G), 19-days gestation (19G), 2-days lactation (2L), 17-days lactation (17L), and 5-days postlactation (5pL).

most prominent protein in the microsomes. Component E increases moderately relative to G after conception and then remains at this level throughout gestation and lactation, declining somewhat again to its virgin level after weaning. (*b*) Components D and F have very low prepartum levels, but by 2 days after parturition and throughout lactation they occur as major proteins. By 5 days after weaning they are reestablished at their prepartum levels. (*c*) The remaining five components (A, B, C, H, and I) are most prominent during the prepartum period and virtually absent during lactation. Only components A and C of this quintet occur at their prepartum levels by 5 days after weaning.

These proteins, except for components H and I, also comprise the principal proteins of the rough vesicles (vesicles with a density greater than that of 1.5 M sucrose) in the microsome preparations (Fig. 7). Components H and I appear to be confined primarily to smooth vesicles, as they are among the four most prominent proteins in the vesicles equilibrating in the 1.16–1.19 density range (gradient fractions 9 and 10) (Fig. 8). The relative amounts of components A-G in the rough vesicles change during epithelial differentiation in the same manner as they do in the total microsome fraction.

During virginity, pregnancy, and postlactation the relative amounts of components A, B, C, E, and G do not vary within the density range exhibited by the rough vesicles. The principal protein composition of rough ER during a nonsecretory period thus appears to be independent of ribosome content. During lactation, however, when components D, E, F, and G comprise the major proteins, the pair D and F is more abundant relative to the pair E and G in the rough vesicles of gradient fractions 1 and 2 than in the vesicles of fractions 3–6. The very dense rough vesicles in the 1.30–1.34 density range, which are encountered only during lactation, thus contain to a greater degree than vesicles of lesser ribosome content the two microsomal proteins which also become prominent only during lactation.

There are several phenomena which could account for the appearance of components D and F in the major protein complement of rough vesicles upon the onset of lactation. (*a*) Their appearance could represent a change in the major membrane protein complement of the mammary epithelial rough ER. (*b*) Components D and F could be secretory proteins residing in the lumen

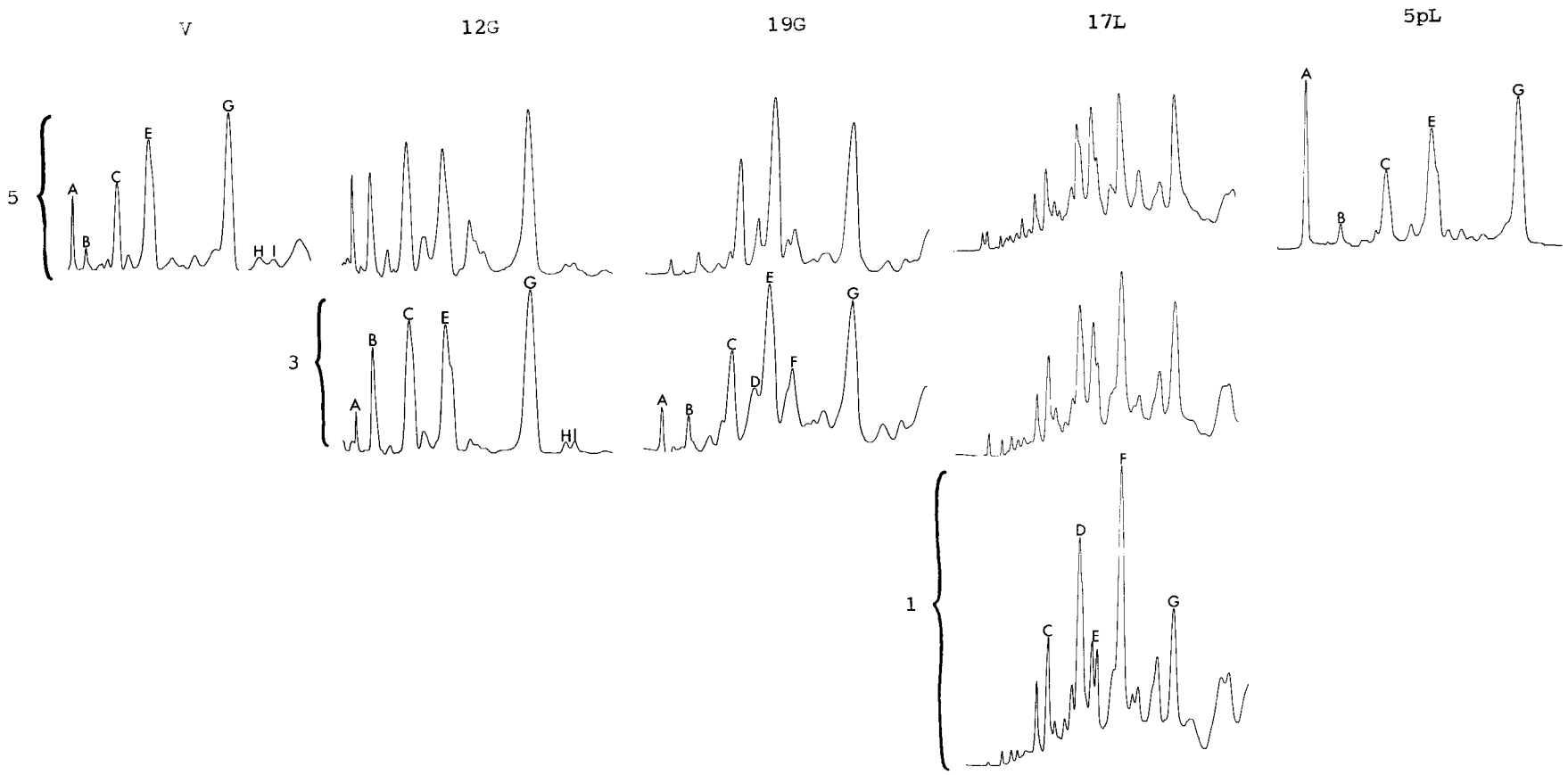

FIGURE 7 Densitometric tracings of electrophoretograms of microsome density subfractions in the lower half of the gradients. The bands representing components D and E have a bipartite nature which is distinctly revealed in the 17-days lactation patterns.

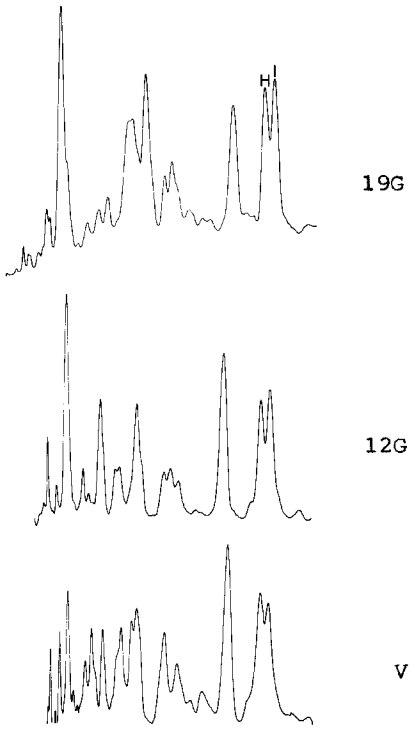

FIGURE 8 Densitometric tracings of electrophoretograms of microsome density subfraction 9 in virgin (V), 12-days gestation (12G), and 19-days gestation (19G) gradients.

of the rough ER which are subsequently retained in the intravesicular space of the rough vesicles after homogenization. (*c*) Both components could be cytosol proteins which become prominent during lactation and appear as components of the rough vesicles due to either ionic adsorption or entrapment upon the vesiculation of the rough ER during homogenization. To test these possibilities, one of two equal portions of a suspension of 17-day lactation rough vesicles in 0.25 M sucrose and 10 mM Tris (pH 7.6) was sonicated with a Branson sonifier (Branson Instruments Co., Stamford, Conn.) at a setting of 2.5 A for five 10-s periods and the other suspended in 0.25 M sucrose, 500 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris (pH 7.6). The suspensions were then centrifuged at 200,000 g_{av} for 3 h. Quantitative gel densitometry of the electrophoretic patterns of the resulting supernate and pellet fractions from the sonicated suspension indicates that 20–25% of the amounts of components D and F have been rendered soluble; both proteins still occur, however, to a major extent in the vesicles (Fig. 9 *a* and *b*). Exposure to a high KCl concentration similarly does not elute components D and F from the rough vesicles; it does result in a partial elution of component E (Fig. 9 *c*). Thus, although both procedures change the relative levels of components D, E, F, and G in the lactation rough vesicles, the four proteins still remain major polypeptide components of the vesicles. The fact, however, that significant amounts of components D and F are released from the rough vesicles upon sonication and that these two components are the most prominent bands in the electrophoretic pattern of the proteins released by sonication indicates that these two proteins are located in both the membranous and intravesicular compartments of the rough vesicles. It is thus not evident whether components D and F are secretory or membrane proteins.

A more general problem is that the microsome preparations are slightly contaminated with mitochondrial fragments, as indicated by the succinate-cytochrome *c* reductase assay. If any major mitochondrial membrane proteins have molecular weights similar to components D and F, then the density-related variation in the relative levels of these two proteins in lactation rough vesicles could be due to a heterogeneous distribution of mitochondrial fragments within the 1.21–1.34 g/ml density range. Accordingly, mitochondria were isolated from 17-days lactation epithelial cells according to the method of Mehard et al. (29) and their equilibrium density distribution in a linear 1.0–2.0 M sucrose gradient determined. Mitochondria isolated from lactation epithelial cells suffer ultrastructural and functional damage during their isolation due to the presence of milk constituents (29); such mitochondria equilibrate principally within the 1.21–1.27 g/ml density range (Fig. 10). Electrophoretic analysis of the mitochondrial fragments contained within this density range reveals that the five most prominent protein bands have molecular weights of 42,000, 50,000, 57,000, 76,000, and 110,000 (Fig. 11). The prominence of presumably components D, F, and G (whose relative levels are similar to those found in lactation rough vesicles) is expected since the mitochondrial preparation is probably somewhat contaminated by vesicles derived from both smooth and rough ER; component E occurs, however, at a relatively low level. The fact that lactation rough vesicles do not have major 50,000 and 110,000 molecular weight protein components suggests that mito-

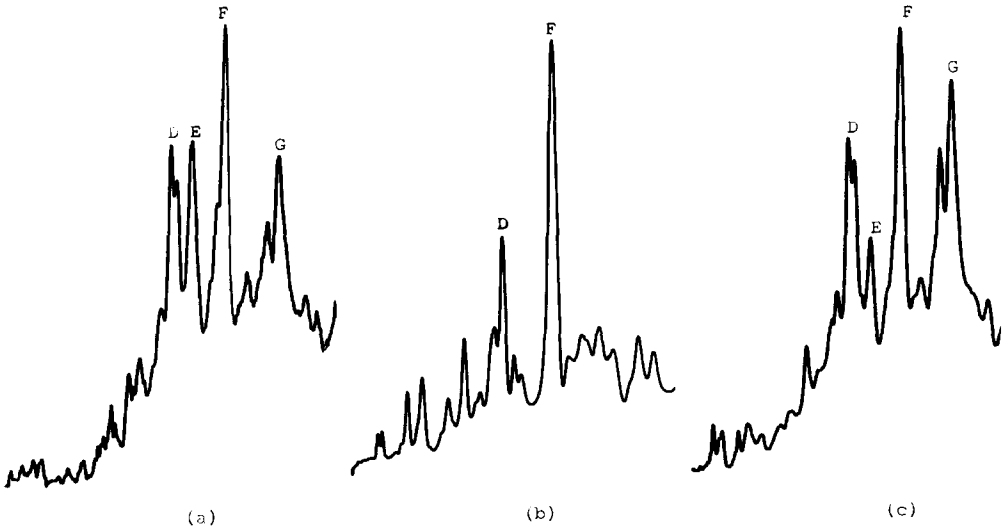

FIGURE 9 Densitometric tracings of electrophoretograms of the pellet (*a*) and supernatant (*b*) fractions obtained after a suspension of 17-days lactation rough vesicles is sonicated and centrifuged as described in the text. The tracings may be quantitatively compared if the scale of tracing (*a*) is multiplied by a factor of three. Densitometric tracing (*c*) is that of the electrophoretogram of 17-days lactation rough vesicles sedimented after exposure to a high KCl concentration as described in the text.

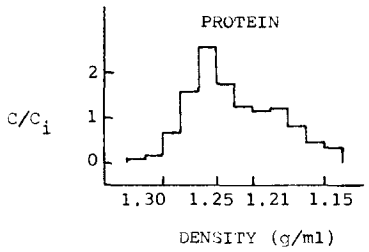

FIGURE 10 Density distribution pattern of mitochondria isolated from 17-days lactation epithelial cells. The mitochondria were suspended in 0.4 ml 0.25 M sucrose and 10 mM Tris (pH 7.6), layered over a 4.6 ml linear 1.0–2.0 M sucrose gradient in 10 mM Tris (pH 7.6), and centrifuged at 230,000 g_{av} for 11 h. The density distribution of protein was then determined.

chondrial fragments are minor contaminants of these vesicles. The results thus indicate that mitochondrial fragments contaminating lactation microsome preparations cause at the very most, because of their low content of component E, the relative level of this component in rough vesicles equilibrating within the 1.21–1.27 density range to appear lower than may actually be the case.

Finally, the contribution of ribosomal proteins to the rough vesicle electrophoretic pattern within

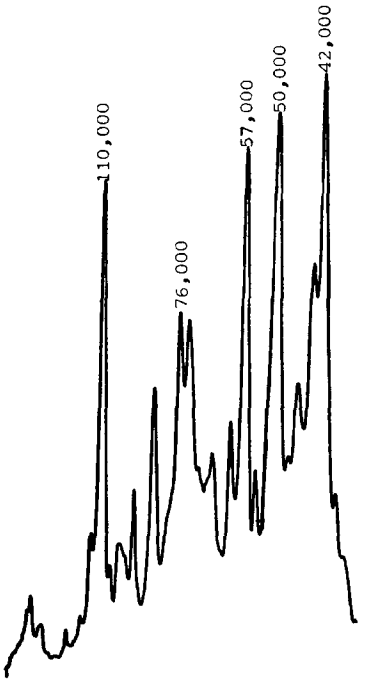

FIGURE 11 Densitometric tracing of the electrophoretogram of mitochondrial fragments contained in gradient fraction 5 of the gradient shown in Fig. 10.

the 40,000–200,000 molecular weight range has been examined. Electrophoretic analysis of ribosomes isolated from 17-days lactation rough vesicles reveals a major 42,000 molecular weight band; there are also two minor 68,000 and 98,000 molecular weight bands (Fig. 12 *a*). If the ribosomes are dissociated by puromycin-KCl treatment and the ribosomal subunits fractionated on a linear sucrose density gradient, all of the protein(s) accounting for the 42,000 molecular weight band are seen to reside within the large ribosomal subunit (Fig. 12 *b* and *c*). It also appears that the puromycin-KCl treatment extracts the small amounts of the 68,000 and 98,000 molecular weight proteins. Quantitative gel densitometry indicates that the large ribosomal subunit 42,000 molecular weight protein(s) account for 15–20% of the total 42,000 molecular weight protein in lactation rough vesicles. This 15–20% figure applies not only when ribosomes are isolated from all the rough vesicles within the 1.21–1.34 density range, but also if the analysis is limited to just the rough vesicles contained within gradient fractions 1 and 2, 3 and 4, or 5 and 6. Thus, there are both membrane and ribosomal proteins which contribute to the 42,000 molecular weight band of the rough vesicle electrophoretic pattern. Since the ribosome content of lactation rough vesicles decreases by approximately a factor of three as one passes from a density of 1.34 to 1.21 g/ml, the results suggest that the density of membranous 42,000 molecular weight protein in lactation rough ER varies directly with the surface density of ribosomes.

Vectorial Discharge of Nascent Polypeptide Chains from Rough Vesicles

The fact that a very dense class of rough vesicles appears in mammary epithelial microsomes at the onset of lactation provides an opportunity to analyze the extent to which these vesicles are derived from rough ER formed after parturition. One of the principal distinctions between prepartum and postpartum rough ER biogenesis is that whereas all the rough ER elements formed during the former period are almost exclusively engaged in the synthesis of intracellular proteins, the rough ER elements formed during the latter period are engaged in both intracellular and milk protein synthesis. Let us assume for the moment that (*a*) rough ER biogenesis occurs via a *de novo* process; that is, that rough ER arises from integral protein synthesizing units consisting of ribosomes, messenger RNAs, and membrane which are formed independently of preexisting rough ER structures, and (*b*) that once formed, these protein synthesizing units maintain their functional integrity for a limited period, perhaps 1 or 2 days. If this is the situation, we would expect that at 1 day after parturition there is a topographical segregation in the cytoplasm of mammary epithelial cells of those nonsecretory rough ER elements formed during late gestation from the nonsecretory and secretory elements newly formed after parturition. It follows then that when 1-day lactation epithelial cells are homogenized, the rough vesicles derived from the nonsecretory rough ER formed during late gestation will exhibit densities ranging from 1.21 to 1.29 g/ml whereas those derived from the newly formed nonsecretory and secretory rough ER will exhibit densities ranging from 1.21 to 1.34 g/ml. 1-day lactation rough vesicles in the 1.30–1.34 density range should therefore have greater secretory activity than those in the 1.21–1.29 range. By 17-days lactation, when all the rough ER elements formed during late gestation have degraded, there should be accordingly less difference between the secretory activities of rough vesicles in the 1.21–1.29 and 1.30–1.34 ranges.

This proposal is subject to direct experimental confirmation as a result of studies Redman and Sabatini (30) and Andrews and Tata (31) have conducted. Their studies demonstrate that rough vesicles isolated from a secretory tissue, such as liver, vectorially transport a greater proportion of the polypeptide chains discharged from the bound ribosomes by puromycin into the lumen of the vesicles than do rough vesicles isolated from a nonsecretory tissue, such as brain (cerebral cortex) or muscle. When such an analysis of the distribution of puromycyl peptides between intra- and extravesicular compartments is conducted on 19-days gestation, 1-day lactation, and 17-days lactation rough vesicles, we find the following: (*a*) More than 90% of the polypeptide chains discharged from late gestation rough vesicles are released into the extravesicular milieu (Table III). (*b*) Chains discharged from 17-days lactation rough vesicles are equally distributed between the intra- and extravesicular compartments. There is no significant difference in the distribution of chains discharged from 17-days lactation rough vesicles in the 1.21–1.29 and 1.30–1.34 density ranges. (*c*)

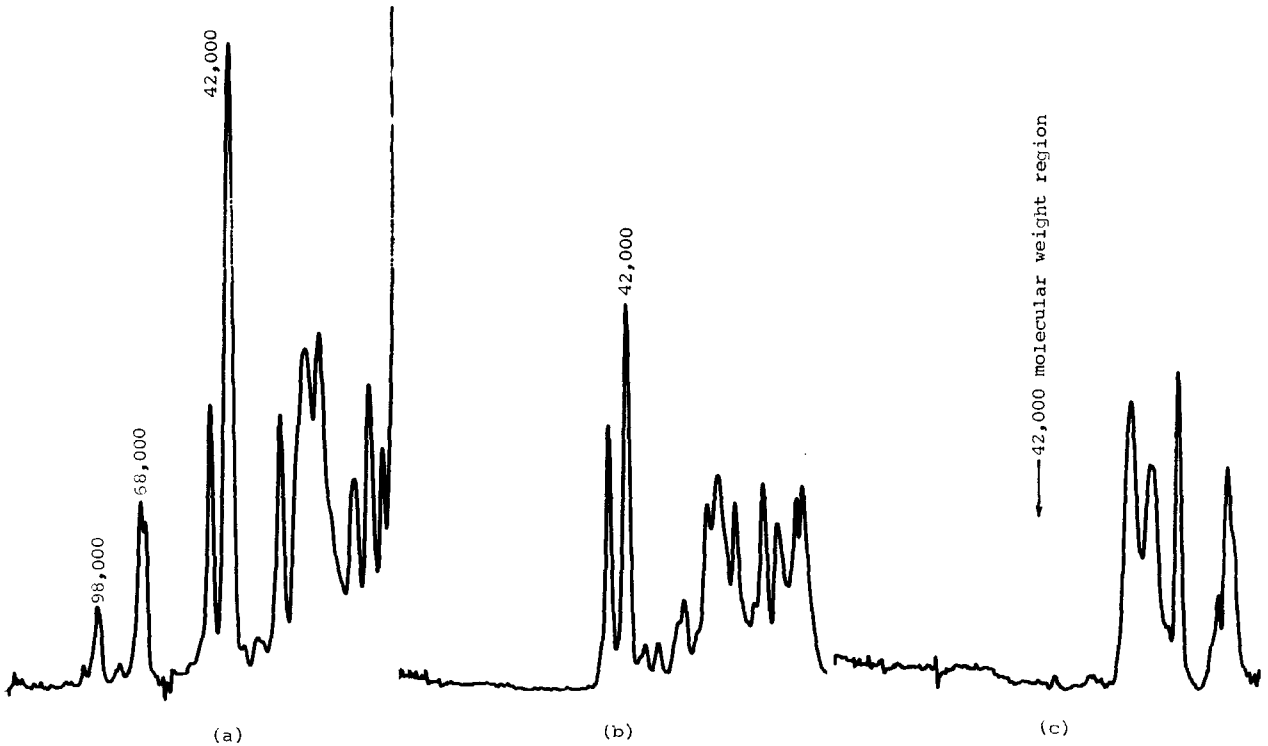

FIGURE 12 Densitometric tracings of electrophoretograms of ribosomes (*a*), large ribosomal subunits (*b*), and small ribosomal subunits (*c*) isolated from 17-days lactation rough vesicles.

TABLE III

Puromycin Effect on the Distribution of In Vitro-Labeled Polypeptides Recovered from Mammary Epithelial Rough Vesicles

Developmental stage and density range of rough vesicles	[^{3}H]Leucine cpm percent			
	Extravesicular supernate	Ribosomes	DOC released	Total precipitable cpm
19-days gestation rough veiscles in the 1.21–1.29 density range				
No puromycin	56	40	4	2,358
Puromycin	69	23	8	1,325
1-day lactation rough vesicles in the 1.21–1.29 density range				
No puromycin	41	49	10	15,479
Puromycin	58	23	19	10,774
1-day lactation rough vesicles in the 1.30–1.34 density range				
No puromycin	22	68	10	31,593
Puromycin	49	23	28	18,105
17-days lactation rough vesicles in the 1.21–1.29 density range				
No puromycin	21	71	8	6,678
Purmocyin	39	18	43	5,040
17-days lactation rough vesicles in the 1.30–1.34 density range				
No puromycin	21	69	10	8,299
Puromycin	40	25	35	5,233

DOC, sodium deoxycholate.

Whereas 36% of the chains released from 1-day lactation rough vesicles in the 1.30–1.34 range are discharged into the intravesicular compartment, only 25% are discharged into the lumen of the vesicles in the 1.21–1.29 range.

Further indication that 1-day lactation rough vesicles in the 1.30–1.34 range have greater secretory activity than those in the 1.21–1.29 range is provided by a comparison of the distribution of completely synthesized proteins (proteins released in the absence of puromycin) between the intra- and extravesicular compartments. The nonsecretory 19-days gestation rough vesicles release 60% of their nascent chains upon 19-min incubation in an amino acid incorporating system, only 10% of which are discharged into the intravesicular compartment. The secretory 17-days lactation rough vesicles, however, release only 30% of their chains, 33% of which are discharged into the intravesicular compartment. 1-day lactation rough vesicles in the 1.30–1.34 range appear identical to the 17-days lactation rough vesicles in that they release only 32% of their chains, 31% of which are discharged into the intravesicular compartment. The 1-day lactation rough vesicles in the 1.21–1.29 range appear similar to the 19-days gestation rough vesicles in that they release 51% of their chains, with only 20% being discharged into the lumen of the vesicles.

DISCUSSION

This study was undertaken to investigate the changes in ribosome content, principal microsomal protein composition, and secretory character of mammary epithelial rough ER during glandular development and regression. The microsome density distribution patterns are consistent with a progressive increase in the average ribosome content of the rough ER during differentiation. This increase is thus strongly correlated with the onset of secretory activity and most likely represents a mechanism by which the protein synthetic capacity of the rough ER is enhanced.

Detailed morphological analysis of rat liver mi-

crosome density subfractions tends to suggest that density dispersion arises principally from the variability in the ribosome content of the vesicles (27). Our analysis of the density distribution patterns indicates, however, that there are other physiochemical factors which are prominent in the determination of the equilibrium densities of vesicles with low RNA contents; these factors probably exert a modulating influence on the equilibrium densities expressed by vesicles having high RNA contents. The large increases in the RNA content of subfractions of equivalent density during gestation and lactation thus arise from two interrelated phenomena: (*a*) The RNA content of a microsomal vesicle does not sharply define its isopycnic density and (*b*) vesicles derived from smooth membrane systems of the mammary epithelium partially equilibrate in the 1.20–1.30 density range, the range where the ribosome-bearing vesicles principally equilibrate. We find that the greater the extent of smooth membrane systems in the epithelium relative to that of rough ER, the lesser the RNA content in this range. The rough ER does not become a major membrane system until midpregnancy, which is when the RNA content of the vesicles in the 1.20–1.30 density range begins to significantly increase. Moreover, the period of maximum rough ER biogenesis, that of the last 9–10 days of gestation, is also the period during which the greatest increases in the RNA content of gradient fractions 3–8 are registered. Afterwards, during lactation, when the proportionate increases of the rough ER content are considerably less, there are, correspondingly, no changes in the RNA content of fractions 6–8 and only moderate increases in fractions 3–5.

Our results also indicate that when mammary epithelial cells at any stage of differentiation are homogenized in an isotonic sucrose solution of low ionic strength, almost all ribosomes (at the minimum 85%) are found to be membrane bound. Wibo et al. (27) similarly concluded that only 10–15% of the ribosomes in their rat liver microsome preparations are membrane free under low ionic strength conditions (3 mM imidazole). Although one might expect almost all the ribosomes to be membrane bound during lactation, electron microscope studies have shown that most of the ribosomes in virgin epithelium are free in the cytoplasm (2). We prepared and dispersed microsomes in media of low ionic strength as this is a condition which minimizes vesicular aggregation (27, 33); it may also, however, be one which generates artefactual ribosome-membrane complexes. When microsomes are prepared in a medium of moderate ionic strength (25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris, pH 7.6), a significant free ribosome population can be recovered in late gestation and lactation preparations. The extensive vesicular aggregation which occurs in such a medium, however, compromises any attempt to disperse the microsomal vesicles according to their individual equilibrium densities (unpublished observations). It has been demonstrated that those ribosomes whose association with membranes remains intact at moderate ionic strengths (0.05) have ionic linkages with the membranes which are disrupted when ionic strengths of the order of 0.5 are approached (32). In agreement with results obtained from studies of liver rough vesicles, 40% of the ribosomes of 17-days lactation rough vesicles are bound solely via such ionic linkages (32). It is possible, therefore, that there are other specific ionic interactions operant only under conditions of low ionic strength.

The finding that lactation rough vesicles in the 1.21–1.29 and 1.30–1.34 density ranges differ in secretory activity at 1-day lactation but not at 17-days lactation indicates the following: (*a*) After parturition, the messenger RNAs specific for milk proteins are incorporated into rough ER elements formed *de novo*. If such incorporation also applies to the other class of messenger RNAs which are introduced into the cytoplasm after parturition; namely, those specific for intracellular proteins, there is a topographical segregation in the cytoplasm of 1-day lactation mammary epithelial cells of those membrane-bound ribosomes synthesizing intracellular proteins specific for late gestation from those synthesizing the intracellular and milk proteins specific for lactation. This proposal is similar to one that Tata and his associates have put forth on the basis of their studies of triiodothyronine stimulation of protein synthesis in bullfrog tadpole hepatocytes (34). (*b*) At 17-days lactation, the proportion of ribosomes among the rough ER elements bearing very high surface densities of ribosomes which is synthesizing milk proteins is the same as that of the ribosomes among the less heavily ribosome-bearing rough ER elements. During lactation the average ribosome content of the rough ER elements synthesizing intracellular proteins is the same as that of the rough ER elements engaged in milk protein synthesis. (*c*) There is no correlation between the

contents of microsomal components D and F in mammary epithelial rough vesicles and the extent of secretory activity. It appears that this postparturitive change in the microsomal protein composition of the rough ER does not have anything to do with the change in secretory character.

Concerning the problem of whether components D and F are secretory or membrane proteins, our finding that these two proteins remain major polypeptide components of lactation-stage rough vesicles after either sonication or elution with 0.5 M KCl agrees with the results of a study recently published by Keenan and Huang (35). They have analyzed by SDS-gel electrophoresis the protein composition of lactation-stage bovine mammary gland rough vesicles washed by the method of Meldolesi et al. (36) to remove adsorbed and intravesicular proteins. Although they count every band in their electrophoretic patterns as representing a major microsomal protein and thus find eight major proteins within the 40,000–80,000 molecular weight range, their analysis concurs with ours in that there are four major microsomal proteins with molecular weights of 74–76,000, 68,000, 57,000, and 42–45,000 in lactation-stage mammary gland rough vesicles. The two most prominent proteins in their electrophoretic patterns have molecular weights of 57,000 and 68,000. They did not determine, however, the extent to which the major microsomal proteins were removed by the washing procedure. Consideration of our results with those of Keenan and Huang (35) indicates that components D and F are not confined to the membranous compartment of mammary epithelial rough vesicles to the same extent as are the other major microsomal proteins. Components D and F may therefore be secretory proteins whose transfer through the membrane into the lumen of the rough ER is slower than that of other milk serum proteins.

As mentioned in the Introduction, there is extensive rough ER accumulation in mammary epithelial cells during the latter half of gestation (2). We might have expected that if the membrane proteins being synthesized during this period are directly incorporated into membranous structures upon their release from ribosomes, that the proportion of puromycyl peptides released from 19-days gestation rough vesicles upon treatment with sodium deoxycholate would be greater than that which is observed. The data suggest that membrane proteins may, in fact, be released into the cytosol and are subsequently incorporated into membranous elements at a locale different from their site of synthesis.

Finally, the finding that there are both ribosomal and membranous 42,000 molecular weight proteins in lactation-stage rough vesicles raises the question of whether there are any 42,000 molecular weight proteins common to both structures. We are presently attempting to determine whether any ribosomal 42,000 molecular weight proteins comigrate with any of the membranous 42,000 molecular weight proteins in polyacrylamide gel systems which fractionate proteins according to both molecular weight and charge.

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