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Lactose and major milk proteins are present in secretory vesicle-rich fractions from lactating mammary gland

(α_{s1}-casein/β-casein/α-lactalbumin/β-lactoglobulin/Golgi apparatus)

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ABSTRACT **Preparations enriched in apparently intact secretory vesicles were isolated from homogenates of lactating rat and bovine mammary tissue by differential and density gradient centrifugation in isoosmotic media. Morphologically, these preparations consisted nearly entirely of vesicles of varying sizes, at least some of which contained casein micelles. Endoplasmic reticulum vesicles, Golgi apparatus cisterna and dictyosomes, mitochondria, peroxisomes, lysosomes, and nuclei were not observed in secretory vesicle-rich fractions. Vesicle preparations were enriched in lactose relative to total membrane fractions from mammary gland. The galactosyltransferase of lactose synthase (UDPgalactose: D-glucose 4-β-galactosyltransferase, EC 2.4.1.22) was also present in secretory vesicle preparations. α_{s1}- and β-caseins, α-lactalbumin, and β-lactoglobulin, the major secretory proteins of differentiated mammary epithelial cells, were identified as constituents of vesicle-rich fractions from bovine mammary gland. These observations suggest that the major carbohydrate and major proteins of milk are compartmentalized into secretory vesicles and are secreted by exocytotic fusion of secretory vesicles with the apical plasma membrane.**

Much progress has been made in isolation of secretory vesicles from a number of tissues including pancreas (1, 2) liver (3, 4), parotid gland (e.g., ref. 5), adrenal gland (e.g., ref. 6), and certain plant materials (e.g., ref. 7). Membranes and contents of these vesicles have been characterized both morphologically and biochemically. Vesicles from the above tissues are tightly packed with contents and, presumably because these constituents are not osmotically active, these vesicles are relatively small, unswollen, and thus stable during homogenization and centifugal isolation. In contrast, secretory vesicles in lactating mammary epithelial cells are swollen and distended (e.g., refs. 8–12). Mammary secretory vesicles are exceptionally fragile in conventional homogenization and density gradient centrifugation procedures and have thus far resisted all attempts at isolation (discussed in refs. 11–13).

The exact nature of the content of secretory vesicles in differentiated mammary epithelial cells is unknown, but morphological observations have revealed the presence of micellar caseins in these vesicles (e.g., refs. 8–12). In addition there is indirect evidence for the presence of lactose, water, and the noncasein protein α-lactalbumin in these vesicles (11–16) and indications that calcium (17), citrate (18), and potassium (19) are also concentrated in vesicles. Direct knowledge of the content of mammary secretory vesicles is crucial to further understanding of the cellular discharge of milk. Milk lipids are secreted in the form of triglyceride-rich globules that are extruded from cells by envelopment in apical plasma membrane (for reviews, see refs. 13, 20, 21). It has been hypothesized that secretory vesicles are responsible for secretion of the serum phase (milk minus the lipid globules) of milk (13, 15, 21, 22).

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In addition to allowing a test of this hypothesis directly, isolation of mammary secretory vesicles would also allow a direct test of the endomembrane hypothesis which predicts that secretory vesicle membranes are apical plasma membrane-like (23) and, in mammary epithelial cells, replenish apical plasma membrane expended in envelopment of lipid globules (13, 21). Because it has not been possible to isolate apical plasma membrane from pancreas or liver, this part of the endomembrane hypothesis has not been tested (for discussions, see refs. 24, 25). The unique mechanism by which milk lipid globules are secreted provides a ready source of apical plasma membrane (for reviews, 13, 15, 20, 21) that can be compared with secretory vesicle membrane.

In this communication we show that secretory vesicle-rich fractions can be isolated from homogenates of lactating mammary gland and that these vesicles are enriched in lactose and the galactosyltransferase of lactose synthase (UDPgalactose: D-glucose 4-β-galactosyltransferase, EC 2.4.1.22). In addition, we show that the major caseins and noncasein (whey) proteins of milk are present in these vesicles.

MATERIALS AND METHODS

Tissue Fractionation. Mammary tissue was collected from Sprague–Dawley rats, nursing at least seven pups, between the 8th and 12th days of lactation. Inguinal mammary glands were removed, dissected free of large connective and adipose tissue fragments, finely minced with surgical scissors, and transferred to ice-cold homogenization medium. Bovine mammary tissue was collected from lactating Holstein cows at slaughter, transported to the laboratory on ice, and otherwise treated as above.

Minced tissue was homogenized in a salt solution formulated to approximate the composition of cow's milk ultrafiltrate (26) and containing 14 mM 2-mercaptoethanol and 1–3% Ficoll. After filtration the homogenate was clarified by brief centrifugation at 1500 × *g* at 2°. The supernatant was layered over a Ficoll/milk salt solution of density 1.05 g/ml; after centrifugation the material banding at the clarified homogenate/Ficoll interface was collected and analyzed as the secretory vesicle fraction. Full details of this procedure as well as biochemical and morphological characterization of the vesicle-rich fraction will be published separately. In some cases, rats received two intraperitoneal injections, at 3 and 1.5 hr before sacrifice, of 5 mg of colchicine; this increased subsequent yield of secretory vesicles (11, 12). The pellets that collected in centrifuge tubes during secretory vesicle isolation on Ficoll gradients were collected and analyzed for lactose content and will be referred to as the "vesicle-depleted particulate fraction." Total particulate fractions were obtained by centrifugation of clarified homogenates at 120,000 × *g* (maximum) for 60 min at 2°.

Analytical Methods. Protein was determined with the Folin phenol reagent with bovine serum albumin as standard (27).

Lactose was measured by the glucose oxidase method after hydrolysis with β-galactosidase (11). Values for lactose were corrected for the endogenous glucose content of the fractions (11). Electrophoresis was performed in 8% polyacrylamide gels containing sodium dodecyl sulfate according to Weber and Osborn (28). Electrophoretic separations were also made in 4 M urea/polyacrylamide gels according to Groves and Kiddy (29). Caseins were stained with amido black (29) and noncasein proteins, with Coomassie blue (28). Prior to fixation and electrophoretic separation, the putative caseins and whey proteins in bovine secretory vesicles were crudely fractionated. Vesicle preparations were suspended in and dialyzed against distilled water and subjected to several cycles of freezing and thawing. After the pH was adjusted to 4.6 with HCl, the precipitated material was collected by centrifugation at 8000 × *g* for 15 min and analyzed as the casein fraction (30); supernatants were recovered, concentrated, and analyzed as the whey protein fraction.

Galactosyltransferase Assay. Lactose synthase activity was assayed as described (31). Complete reaction mixture contained, in a final volume of 0.1 ml, the following: 20 mM Tris·HCl (pH 7.4), 20 mM $MnCl_2$, 20 μg of α-lactalbumin, 20 mM D-glucose, 0.5% Triton X-100, 0.25 mM UDP-galactose (15–25 × 10^5 cpm/μmol), and 50 μg of fraction protein. Reactions were stopped by addition of 50 μl of 200 mM EDTA, the product was separated from UDP-galactose on an ion exchange resin (32), and radioactivity was determined with a liquid scintillation counter. Controls without added acceptor were included to correct for hydrolysis of UDP-galactose.

Electron Microscopy. Secretory vesicle-rich fractions and vesicle-depleted particulate fractions were fixed for 1 hr at room temperature in 1% paraformaldehyde/3% glutaraldehyde/0.1 M sodium phosphate, pH 7.0 (33). Fixed material was collected by centrifugation and the pellets were cut into small pieces along the axis of sedimentation, postfixed for 1 hr in 1% osmium tetroxide, dehydrated, and flat-embedded in the epoxy resin of Spurr (34). Thin sections were cut along the axis of sedimentation and counterstained with uranyl acetate and lead citrate. Sections were systematically examined from top to bottom in a Philips EM 300 electron microscope operated at 60 kV. Small blocks of mammary tissue were fixed and processed for microscopic examination as described (11, 12).

RESULTS

Secretory vesicles in epithelial cells of lactating mammary gland are large and swollen (Fig. 1*A* is representative), presumably due to the presence of lactose, which is osmotically active and would pull water into the vesicles (11, 12, 14, 15). These vesicles are exceptionally fragile. We have found that mammary secretory vesicles can be kept intact by very gentle disruption of tissue in a salt solution that mimics bovine milk ultrafiltrate in composition; addition of Ficoll to this homogenization medium appears to aid in stabilizing vesicles. Vesicle-enriched preparations could be obtained by centrifugation on Ficoll density gradients (Fig. 1 *B* and *C*). These fractions consisted predominantly of vesicles of varying sizes, some of which contained dense, electron-opaque granules morphologically recognizable as casein micelles. At higher magnification these vesicles were observed to be bounded by a single, unit-like membrane, approximately 90 Å thick, which appeared to be intact (Fig. 1*C*). Many vesicles contained filamentous strands or casein micelles. This filamentous material may represent premicellar caseins (10, 35). Other morphologically recognizable subcellular fractions—such as nuclei, mitochondria, Golgi apparatus dictyosomes, peroxisomes, lysosomes, and endoplasmic reticulum vesicles—were not observed in these preparations. Identical observations were made with rat (Fig. 1) and bovine (not shown) vesicle preparations. Enzymatic and compositional analyses, full details of which will be published subsequently, verified these morphological observations. Secretory vesicle fractions resembled plasma membranes and milk fat globule membrane in phospholipid composition and in specific activities of enzymes normally found in cell surface membranes.

When measured on a protein basis, lactose was concentrated about 7-fold in secretory vesicle fractions relative to total particulates (Table 1). Vesicle-depleted particulates contained slightly less lactose than did total particulate fractions. On morphological examination, vesicle-depleted particulates were observed to be heterogeneous with an abundance of rough endoplasmic reticulum vesicles, free casein micelles, and variable amounts of small, smooth membrane vesicles; recognizable secretory vesicles were not seen in these preparations. Total homogenates were high in lactose, most probably due to the large amounts of milk trapped in alveolar spaces and the ductal system of the mammary tissue. By our method, lactose content of rat milk was found to be 32 ± 4 mg/ml (mean ± SD, samples from four animals), in accord with literature values of 2.5–3.5% for the lactose content of rat milk (36).

The galactosyltransferase of lactose synthesis, a marker enzyme for Golgi apparatus from mammary gland (21, 37), was also present in secretory vesicle preparations (249.0 ± 9.1 nmol/hr per mg of protein, mean ± SD, fractions from four animals). Specific activity of this transferase was enriched about 4-fold in secretory vesicle fractions relative to total homogenates (63.6 ± 2.1 nmol/hr per mg of protein, mean ± SD, four animals). This is lower than the approximately 16-fold enrichment of this activity in Golgi apparatus fractions from lactating rat mammary gland (37). Part of this diminution in specific activity from Golgi apparatus to secretory vesicles could be due to a higher concentration of secretory protein in vesicles. Alternatively, the activity of the transferase may be diminished as it is excised from the membrane, apparently by a protease (cf. refs. 38 and 39). This galactosyltransferase is also present in rat liver Golgi apparatus (24, 32) and secretory vesicle fractions (3, 24). Although in rat liver this transferase is enriched in immature secretory vesicles relative to Golgi apparatus (3), its specific activity appears to be diminished in mature secretory vesicles (24).

Because the major proteins of bovine milk have been extensively characterized, vesicles from bovine mammary gland were used exclusively for identification of content proteins (30). When intact secretory vesicle-rich fractions were prepared directly and the proteins separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, bands comigrating with major milk caseins and whey proteins were evident (Fig. 2*A*). However, the polypeptide pattern of secretory vesicles were complex, as would be expected if both membrane and secretory proteins were present. In addition, the major caseins of bovine milk (α_{s1}, M_r 23,500, 45–55% of total milk protein;

Table 1. Concentration of lactose in secretory vesicles from rat mammary gland

Fraction	Lactose content, μg/mg protein*
Secretory vesicles	122 ± 35
Homogenate	94 ± 7
Total particulate	18 ± 4
Vesicle-depleted particulate	13 ± 1

* Values are means ±SD for determinations with preparations from four animals.

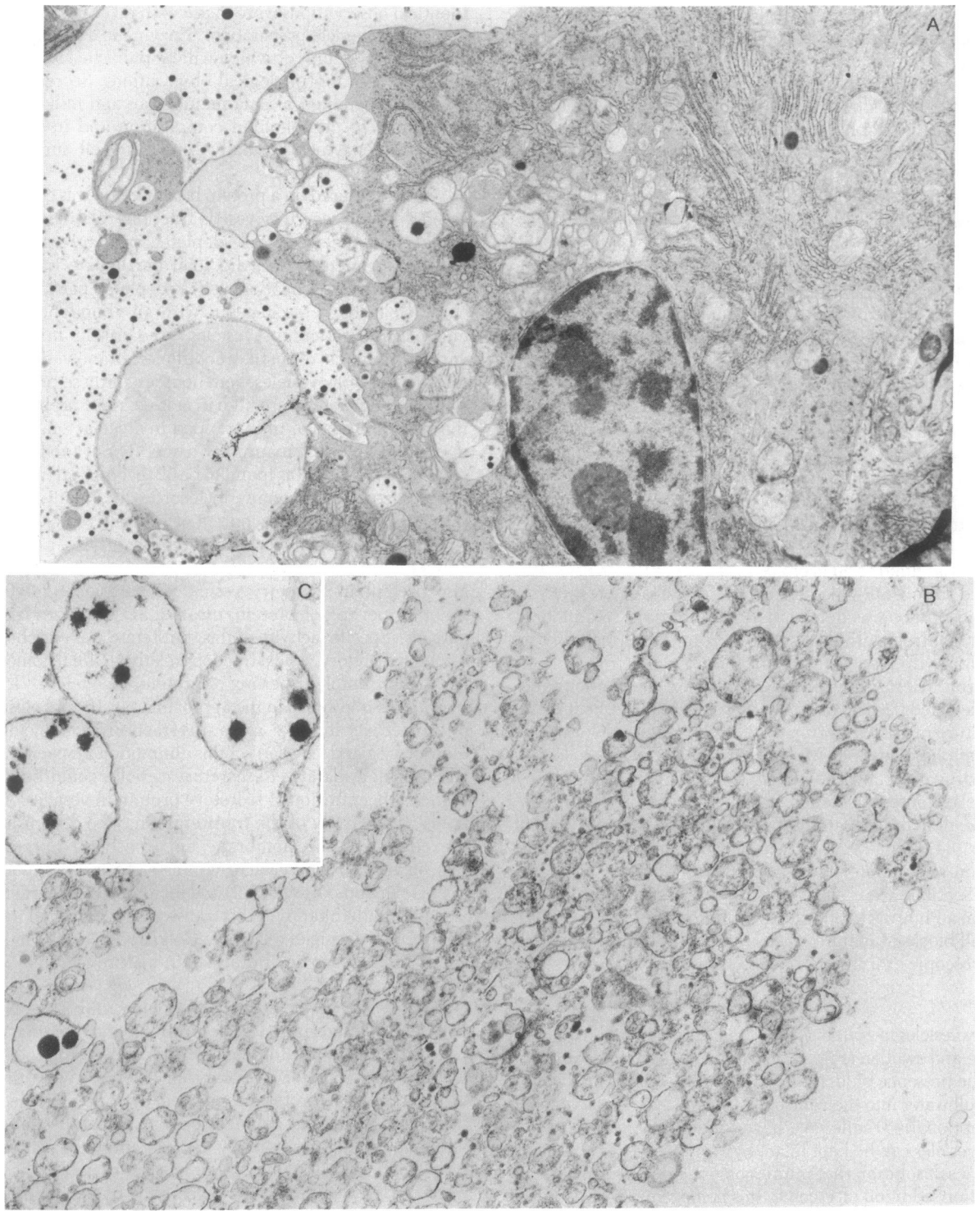

FIG. 1. Electron micrographs of rat mammary epithelial cell at the 10th day of lactation (*A*) and of secretory vesicle-rich fractions isolated from lactating rat mammary gland (*B*) and(*C*). Note the swollen, distended appearance of secretory vesicles containing electron-dense granules (casein micelles) in intact cells (*A*) and in the isolated fraction (*C*). (*B*) Survey micrograph of a typical vesicle-rich preparation. (*A*, ×8500; *B*, ×15,000; *C*, ×37,000.)

β, M_r 24,000, 25–35%; and κ, M_r 19,000, glycosylated, 8–15%) (30) do not separate well from each other on sodium dodecyl sulfate/polyacrylamide gels. For this reason, a crude separation of caseins was performed and the putative casein-containing fraction was separated on urea/polyacrylamide gels (Fig. 2*B*). Bands that comigrated with α_{s1}- and β-caseins were the major polypeptides detected, and the relative ratio of these constituents was similar to the ratio of these proteins in milk. κ-Casein was not evident in these patterns; because κ-casein stains poorly (see, for example, the reference casein gel in Fig. 2*B*), it would not be detected at these concentrations. When vesicle contents were iodinated with ^{125}I, autoradiograms of gels revealed the presence of a constituent with κ-casein mobility (not shown). Iodinated secretory vesicle contents were treated with rabbit antisera to bovine caseins and the immunoprecipitates were separated by electrophoresis. Autoradiograms revealed the presence of constituents migrating with α_{s1}-, β-, and κ-caseins (not shown).

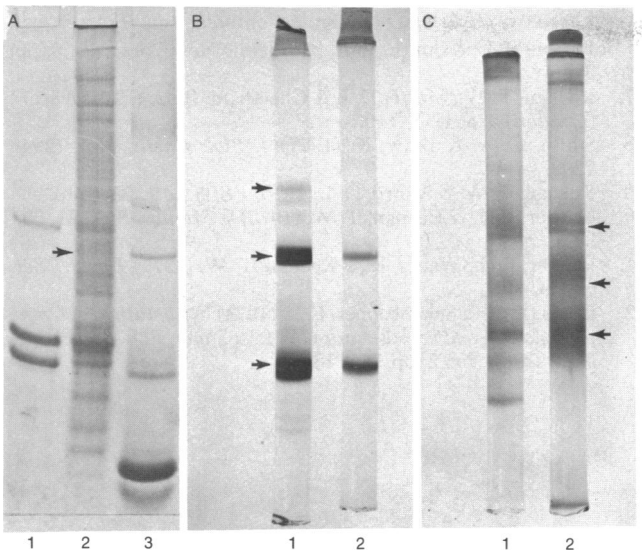

FIG. 2. Electrophoretic separation of proteins in secretory vesicle-rich fractions from bovine mammary gland. (*A*) Sodium dodecyl sulfate/polyacrylamide gel stained with Coomassie blue. Lanes: 1, milk caseins; 2, secretory vesicle total proteins; 3, milk whey proteins. Arrow, position of bovine serum albumin. (*B*) Urea/polyacrylamide gels stained with amido black. Lanes: 1 milk caseins; 2, the acid-precipitable fraction from lysed secretory vesicles. Arrows, positions of κ-casein, β-casein, and α_{s1}-casein from top to bottom, respectively. (*C*) Acid-nonprecipitable protein fraction from lysed secretory vesicles (lane 1) and milk whey proteins (lane 2). Arrows, positions of bovine serum albumin, α-lactalbumin, and β-lactoglobulin from top to bottom, respectively. Gels stained with Coomassie blue.

Polypeptides migrating with α-lactoglobulin, β-lactalbumin, and bovine serum albumin were observed in both dodecyl sulfate/polyacrylamide (Fig. 2*A*) and urea/polyacrylamide (Fig. 2*C*) gel patterns of the acid-nonprecipitable fraction from bovine secretory vesicles. The polypeptides corresponding in mobility to α-lactalbumin and β-lactoglobulin were present in about the same ratio as they are in milk. The major polypeptide of bovine milk fat globule membrane, a highly hydrophobic, glycosylated protein, comigrates with bovine serum albumin in dodecyl sulfate gels (e.g., refs. 40, 41). Thus, we cannot identify this particular polypeptide in vesicle fractions as bovine serum albumin; it may well be a membrane polypeptide. Immunoprecipitates obtained when antisera to bovine whey were added to ^{125}I-labeled vesicle contents contained constituents migrating with α-lactalbumin and β-lactoglobulin, as detected by autoradiography (not shown).

DISCUSSION

Isolation of intact secretory vesicles from mammary gland was critically dependent on gentle homogenization and use of media of proper tonicity. Simulated milk ultrafiltrate was chosen on the basis of the suggestion that vesicle content is similar to milk serum in composition (14, 22). Fractions enriched in apparently intact secretory vesicles can be obtained from lactating mammary tissue of either rat or bovine by using this method.

Lactose is concentrated in secretory vesicles relative to other mammary membrane vesicles. This is direct confirmation of previous indirect demonstrations that lactose is present in secretory vesicles (11, 12, 16) and suggests that secretory vesicles may be a major route for cellular discharge of this sugar which is the major carbohydrate in milk of numerous species (36). The presence of an osmotically active compound such as lactose would cause water to be drawn into vesicles and would account for their swollen appearance. That the galactosyltransferase of lactose synthesis, a known Golgi apparatus marker enzyme (21, 37), is present in vesicles confirms that they originate from Golgi apparatus cisterna. The presence in vesicles of this enzyme as well as the specifier protein α-lactalbumin (e.g., ref. 42) suggests that lactose synthesis may continue during formation of vesicles and their migration to the apical cell surface.

That α_{s1}- and β-caseins are present in vesicle fractions confirms numerous morphological observations that casein micelle-like particles are present in intracellular secretory vesicles. The presence of polypeptides coelectrophoresing with α-lactalbumin and β-lactoglobulin demonstrates that major milk whey proteins are also contained in secretory vesicles.

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