REDOX CONSTITUENTS IN MILK FAT GLOBULE MEMBRANES AND ROUGH ENDOPLASMIC RETICULUM FROM LACTATING MAMMARY GLAND

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ABSTRACT

Milk fat globule membranes (MFGM) and rough endoplasmic reticulum (RER) membranes were isolated from milk and lactating mammary gland from the cow and were characterized by biochemical and electron microscope methods in terms of gross composition (proteins, phospholipids, neutral lipids, cholesterol, RNA, and DNA) and purity. Both fractions contained significant amounts of a b-type cytochrome with several properties similar to those of cytochrome b_5 from liver, as well as a rotenone-insensitive NADH- and NADPH-cytochrome c reductase. The b-type cytochrome content in the apical plasma membrane-derived MFGM was of the same order of magnitude as it was in RER membranes. It was characterized by a high resistance to extraction by low- and high-salt concentrations and nonionic detergents. MFGM contained much more flavin and much higher activities of xanthine oxidase than the RER membranes. The same redox components were found in MFGM and mammary RER from women, rats, mice, and goats, but in absolute contents great differences between the species were noted.

The cytochromes described here differed from liver cytochrome b_5 in some spectral properties. The α-band of the reduced hepatic cytochrome b_5 is asymmetric with a maximum at 555 nm that is split into two distinct peaks at low temperatures. The α-band of the b-type cytochromes from MFGM and mammary RER appears as one symmetrical peak at about 560 nm that is not split at low temperatures. When treated with cyanide, MFGM and mammary microsomes showed difference spectra of a reduced b-type cytochrome. Under the same conditions, liver microsomes gave a completely different spectrum.

These findings demonstrate the presence of a b-type cytochrome and associated redox enzymes in MFGM, i.e., a derivative of the apical cell surface membrane that is regularly used for envelopment of the milk fat globule during secretion.

In 1955 Bailie and Morton (3) reported the discovery of a hemochromogen in a particulate fraction from bovine milk. This hemochromogen had spectral properties similar to those of the cytochrome b_5 reported from microsomal membranes of rat liver and bovine mammary gland. While

Bailie and Morton assumed that the particles associated with the milk fat globules (MFG) were derived directly from microsomal, i.e., endoplasmic reticulum (ER), membranes of mammary epithelial cells (3, 4), further morphological and biochemical studies have shown that the milk fat globule membrane (MFGM) is budded from the apical plasma membrane during milk lipid globule secretion (e.g. 5; for additional references, see 25). Occasionally, minor amounts of ER and other cytoplasmic membrane elements are entrained between the lipid globule and MFGM during secretion (e.g. references 42 and 73). Since cytochromes have so far not been unequivocally demonstrated as constituents of surface membranes of eucaryotic cells (e.g. 21, 35; for controversial data, see references 12 and 70), the occurrence of a cytochrome in MFGM is of special interest. We have studied, by spectrophotometric and enzymatic methods, the redox constituents in MFGM from cow, human, and rat in comparison with rough endoplasmic reticulum (RER) membranes from lactating mammary glands. The data obtained strongly suggest that a b-type cytochrome and NADH- and NADPH-cytochrome c reductase activities, xanthine oxidase, and flavins are present in the MFGM, a derivative of the apical cell surface membrane.

MATERIALS AND METHODS

Materials

Bovine milk was freshly obtained from individual 3- to 6-year-old Holstein or Allgäu cows at a local farm. Goat's milk was obtained from Morrocan dwarf goats. Human milk samples from women in the 1st or 2nd post partum wk were collected from volunteers at the Women's Hospital of the University of Heidelberg (the kind cooperation of Dr. Müller is gratefully acknowledged) with the aid of a milk pump. Rat and mouse milk samples were collected from Sprague-Dawley rats and Naval Medical Research Institute (NMRI) mice during the second week of lactation. The animals were milked by gentle rhythmic suctioning with a special pipette after intraperitoneal injection of oxytocin (50 USP units, Serva Feinbiochemica, Heidelberg, Fed. Rep. of Germany).

Lactating bovine mammary tissue was obtained within a few minutes after death of Holstein cows at a local slaughterhouse. Tissue slices (1-2 cm) or minces were immediately prepared and were kept in ice-cold homogenization medium until fractionation, which was started within 20-30 min after death of the animals. Mammary glands from rats (Sprague-Dawley strain) and mice (NMRI strain) were removed from fully lactating animals killed by cervical dislocation. Small breast biopsy specimens from lactating women suffering from mastitis were taken during surgery at the Women's Hospital of the University of Heidelberg.

All chemicals used were of the highest purity available and were purchased from C. F. Boehringer and Sons (Mannheim, Fed. Rep. of Germany) or Merck A. G., Inc. (Darmstadt, Fed. Rep. of Germany) if not otherwise indicated.

Isolation Procedures

MILK FAT GLOBULE MEMBRANES (MFGM): MFG were prepared from milk samples which had been diluted twice with buffered saline solution (0.15 M NaCl, 2 mM $MgCl_2$, 10 mM Tris-HCl buffer, pH 7.4). After centrifugation at 2,500 g for 15 min at room temperature, the floated cream was collected and washed three times by gentle resuspension and centrifugation in buffered saline. For disruption of MFG, the washed cream was then resuspended in cold medium (75 mM NaCl, 1 mM $MgCl_2$, 5 mM Tris-HCl buffer, pH 7.4) and churned with the top-driven rotating knife homogenizer (E. Bühler, Tübingen, Fed. Rep. of Germany). "Crude MFGM" were then collected from the buttermilk by sedimentation at 100,000 g for 90 min. The pellet was resuspended in 0.3 M sucrose containing 10 mM Tris-HCl buffer, pH 7.4, 70 mM KCl, and 2 mM $MgCl_2$ (TKM). The membrane suspension was layered onto a continuous sucrose gradient made up in TKM (density range from 1.11 to 1.27 g/cm^3) and was centrifuged at 80,000 g for 4 h or overnight (ca. 12 h). The material banding with a buoyant density of 1.18-1.23 g/cm^3 was collected, diluted with TKM, sedimented, and resuspended in the 0.3 M sucrose-TKM medium. This fraction was designated "Purified MFGM." In many experiments, it was further extracted with 10 vol of 1.5 M KCl buffered with 10 mM Tris-HCl, pH 7.4, at 4°C for 4 h. The extracted membrane material was sedimented at 100,000 g for 90 min, resuspended in 0.3 M sucrose-TKM, and designated "Purified MFGM, high salt extracted." The supernatant fluid from this extraction was dialyzed three times against 5 liters of 1 mM Tris-HCl buffer, pH 7.4, and the precipitated material was collected by centrifugation at 100,000 g for 90 min. This subfraction was designated "Water-insoluble high salt extract (isHSE) from MFGM." In recovery experiments, the nonsedimentable material of the high salt extract obtained after dialysis, i.e., the material that did not precipitate, was also analyzed. For this purpose, the supernatant fluid of the last centrifugation step was concentrated by dry dialysis against polyethylene glycol (mean mol wt 40,000) for 3-6 h and was designated "Water-soluble high salt extract (sHSE) from MFGM."

Mg^{++} ions were present in the isolation media only to directly compare the MFGM and RER preparations. Control experiments revealed identical results when Mg^{++} ions were omitted during the preparation of the

MFGM, even when the media were supplemented with 2 mM EDTA.

ROUGH ENDOPLASMIC RETICULUM (*RER*): For preparation of RER fractions from lactating mammary gland, large strands of connective tissue were removed. The remaining tissue was washed with buffered saline solution (see above), homogenized with the rotating knife homogenizer in 0.3 M sucrose-TKM, and filtered several times through four layers of fine-mesh cheese-cloth. The filtrate was homogenized with a glass-Teflon Potter-Elvehjem device and centrifuged at 2,500 g for 15 min. The supernate was centrifuged twice at 10,000 g for 10 min and the pellets obtained were designated "Crude mitochondrial fraction." "Total RER membranes" were collected from this supernate by centrifugation at 100,000 g for 90 min and resuspended in 0.3 M sucrose-TKM medium. The following microsomal subfractions were prepared by methods outlined for MFGM subfractionation: "RER, high salt extracted," isHSE from RER, and sHSE from RER.

Mitochondrial contamination was further reduced by a modified procedure described for the isolation of rat liver RER (49): The NaCl-washed mammary tissue was transferred to a medium containing 0.5 M sucrose, 1% dextran, 5 mM $MgCl_2$, and 40 mM Tris-maleate buffer, pH 6.5 (1 g tissue per ml medium), and homogenized with the rotating knife device (five times for 5 s at maximal speed). The homogenate was then filtered through cheesecloth, treated with the Potter-Elvehjem homogenizer, and centrifuged at 2,500 g for 15 min. The supernate obtained was diluted with 5 mM $MgCl_2$, 40 mM Tris-maleate buffer, pH 6.5, to a final concentration of 0.3 M sucrose, followed by a centrifugation at 10,000 g for 10 min. This supernate was mixed with a 3 M sucrose solution (5 mM $MgCl_2$, 20 mM Tris-maleate, pH 6.5) to a final concentration of 2 M sucrose. The suspension was layered on the bottom of a centrifuge tube (SW 27 rotor, Beckman ultracentrifuge, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and over-layed with 8 ml each of 1.8 M, 1.5 M, and 1.3 M sucrose solution (same buffer) and centrifuged at 80,000 g for 4 h. The material banded at the 1.8 M/2.0 M sucrose interface was collected, resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.3 M sucrose, and was centrifuged at 100,000 g for 1 h. It was finally resuspended in 0.3 M sucrose-TKM and designated "Purified RER."

In some experiments, the fractions obtained were further extracted with Triton X-100. The amount of protein was 40 mg in 5 ml of 0.3 M sucrose-TKM. Triton X-100 was slowly added with stirring to a concentration of 0.5-5 mg per mg protein, and the mixture was kept in an ice-bath for 2 h. Samples were then centrifuged at 120,000 g for 1 h, and the pellets and supernates were analyzed separately.

Chemical Analyses

Protein was determined by the procedure of Lowry et al. (43) with bovine serum albumin as standard or, in some control experiments, with a modified Nessler reaction (66). In fractions that had been treated with Triton X-100, protein was estimated by the biuret method (29). Lipids were extracted, and lipid phosphorus was analyzed as described elsewhere (22, 38). Phospholipids and neutral lipids were separated by thin-layer chromatography (38), and the amount of phospholipids relative to neutral lipids was determined from the ratio of the saponified fatty acids of these lipid classes by gas-liquid chromatography (57). Molecular weights of 770 for phospholipids and 840 for triglycerides were assumed. Cholesterol was measured by the method of Clark et al. (13) and protein phosphorus according to Ahmed and Judah (1). Nucleic acids of total milk fat globule fractions were extracted from lyophilized samples with a phenol-cresol mixture in the presence of the ribonuclease inhibitor diethylpyrocarbamate. The nucleic acids were then precipitated with ethanol (for details, see reference 54) and purified by the method of Marmur (44). The RNA and DNA contents were estimated from the spectrum in the range of 230-300 nm after digestion with either pancreatic RNase or DNase. Flavins were extracted from the membranes with trichloroacetic acid before and after digestion with trypsin and determined from the spectra of their reduced and reoxidized forms in the range of 400-530 nm (61).

Determination of Cytochromes

The cytochrome content of the fractions was estimated as previously described (36). Experimental details are given in the legends to the figures. For carbon monoxide and cyanide difference spectra, it was important that traces of nonspecifically membrane-attached hemoglobin were carefully removed. This was effected by resuspension i in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.3 M sucrose, and resedimentation of the membrane fraction at 100,000 g for 1 h. The membranes were suspended in a medium containing 50% glycerol and 0.2 M potassium phosphate buffer, pH 7.4, and spectra were recorded at 20°C or at −196°C. In the latter case, the samples were devitrified before use (30), using cuvettes with 1- or 2-mm optical paths. A dual wavelength spectrophotometer DW 2 (American Instrument Co., Aminco, Travenol Laboratories, Inc., Silver Spring, Md.) was used for the spectral analyses.

Enzyme Assays

NADH-cytochrome c reductase, with and without rotenone or antimycin (Sigma Chemical Co., St. Louis, Mo.), NADPH-cytochrome c reductase, succinate dehydrogenase, NADH-, NADPH-, and cytochrome c oxidase activities were determined as described previously (36). Xanthine oxidase activity was measured according to Roussos (63), with an absorbance difference between uric acid and hypoxanthine of 11.0 mM^{-1} cm^{-1} at 290 nm. Peroxidase activity was assayed with the guaiacol

test (11). These enzyme activities were recorded with the Aminco DW 2 spectrophotometer; the limit of detection was at an absorbance difference of 2×10^{-4} per minute. Oxygen consumption was measured with a K-IC Oxygraph (Gilson Medical Electronics, Inc., Middleton, Wis.) equipped with a rapidly oscillating platinum electrode and a thermostatically controlled cuvette of 1.5 ml. The N-demethylation of codeine was measured as described by Axelrod (2), and the desaturation of stearic acid and stearyl coenzyme A was determined according to McDonald and Kinsella (46). All enzymes were assayed at various protein concentrations. Control experiments used samples denatured by boiling.

Electron Microscopy

Samples of MFG, MFGM, and RER fractions were fixed for electron microscopy either in suspension or as pellets, and were processed as described (26). Micrographs were taken with a Siemens Elmiskop 101. Quantitation of contaminants was performed by membrane profile tracing as indicated elsewhere (25) and by the stereological procedure of Weibel et al. (72).

RESULTS

### Morphological Characterization of the Fractions

Isolated MFGM fractions consisted mainly of sheetlike fragments of the surface membrane which revealed on one face a fuzzy coat of heavily stained material (Figs. 1 and 2; cf. also references 10 and 39). In contrast to the small (below 10 nm) components of the other (external) side of these membranes, this fuzzy (internal) coat was much larger though variable in thickness (up to 50 nm). Since this thick dense layer is recognized on the inner aspect of the apical surface membrane during the budding process, we conclude that it represents the inner, i.e., originally cytoplasmic surface of the MFGM (for references, see 26 and 74). The association between this internal coat material and the membrane is very stable, and only small amounts of this layer and their constituent proteins can be removed by treatments with low- and high-salt concentrations. The ultrastructure of the

membrane proper was apparently unaltered by the purification and washing procedures, even in the presence of 2 mM EDTA, 0.1% deoxycholate, or Triton X-100. It revealed a typical unit membrane pattern (Fig. 1c). The MFGM fragments varied considerably in size, depending to some extent on the specific disintegration treatment, especially the extent of churning applied, to release membranes from the globules (cf. Figs. 1 and 2).

Since the associated internal coat provides a marker for identifying MFGM fragments (for the association between this coat material and the apical plasma membrane and the intact MFG and the difference between it and the polygonal "bristle" coat of casein secretory vesicles, see references 26 and 74), it was easy to estimate the degree of contamination of this fraction by other membrane profiles. Although some MFG show inclusions of intracellular elements, particularly ER fragments and mitochondria (cf. references 42 and 73), such membranes were virtually absent from our bovine MFGM fractions (Figs. 1 and 2). When we determined the contamination by non-plasma membrane profiles in intact MFG by quantitative morphometry (these values reflect the maximum possible contamination of MFGM fractions derived from the globules), we found a total contamination in bovine samples of less than 3% (Table I). The corresponding values in rat and human milk were markedly higher, particularly with respect to contamination by ER-elements (maximally 17%; Table I), and exhibited a considerable variation among the preparations and the subjects.

The lactating mammary gland is rich in ergastoplasmic arrays of RER (cf. references 26, 34, and 42). A quantitative morphometry of rough microsomal fractions isolated from mammary gland (Fig. 3; cf. reference 40) showed that less than 7% of the total identifiable membrane profiles were in smooth surfaced vesicles or cisternae, including some obviously plasma membrane-derived fragments (Fig. 3); about 2% were in identifiable mitochondrial membranes. The appearance of the

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FIGURE 1 a–c Electron micrographs of purified bovine MFGM fraction. Fig. 1 a presents a survey of the native fraction and illustrates the high purity. The MFGM fraction contains a high proportion of open membrane sheets (lamellae; some are denoted by arrows) of widely different sizes (some up to several μm large). Fig. 1 b and c present the appearance of such membranes at higher magnifications and show the densely stained, thick fuzzy coat layer associated with the internal side of these membranes (arrowheads in Fig. 1 c). Spikelike structures characteristic for the external coat are occasionally revealed on the outer MFGM face. Bars: (a) 0.5 μm and (b and c) 0.1 μm. (a) × 62,000; (b) × 127,000; and (c) × 177,000.
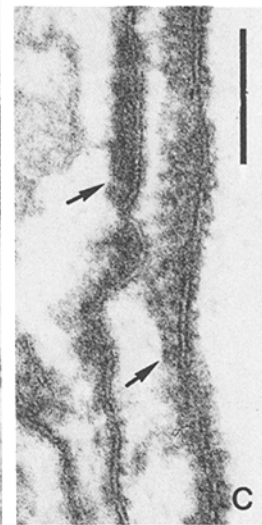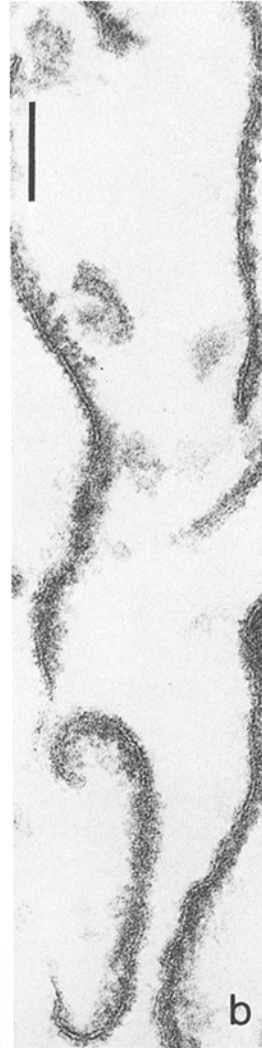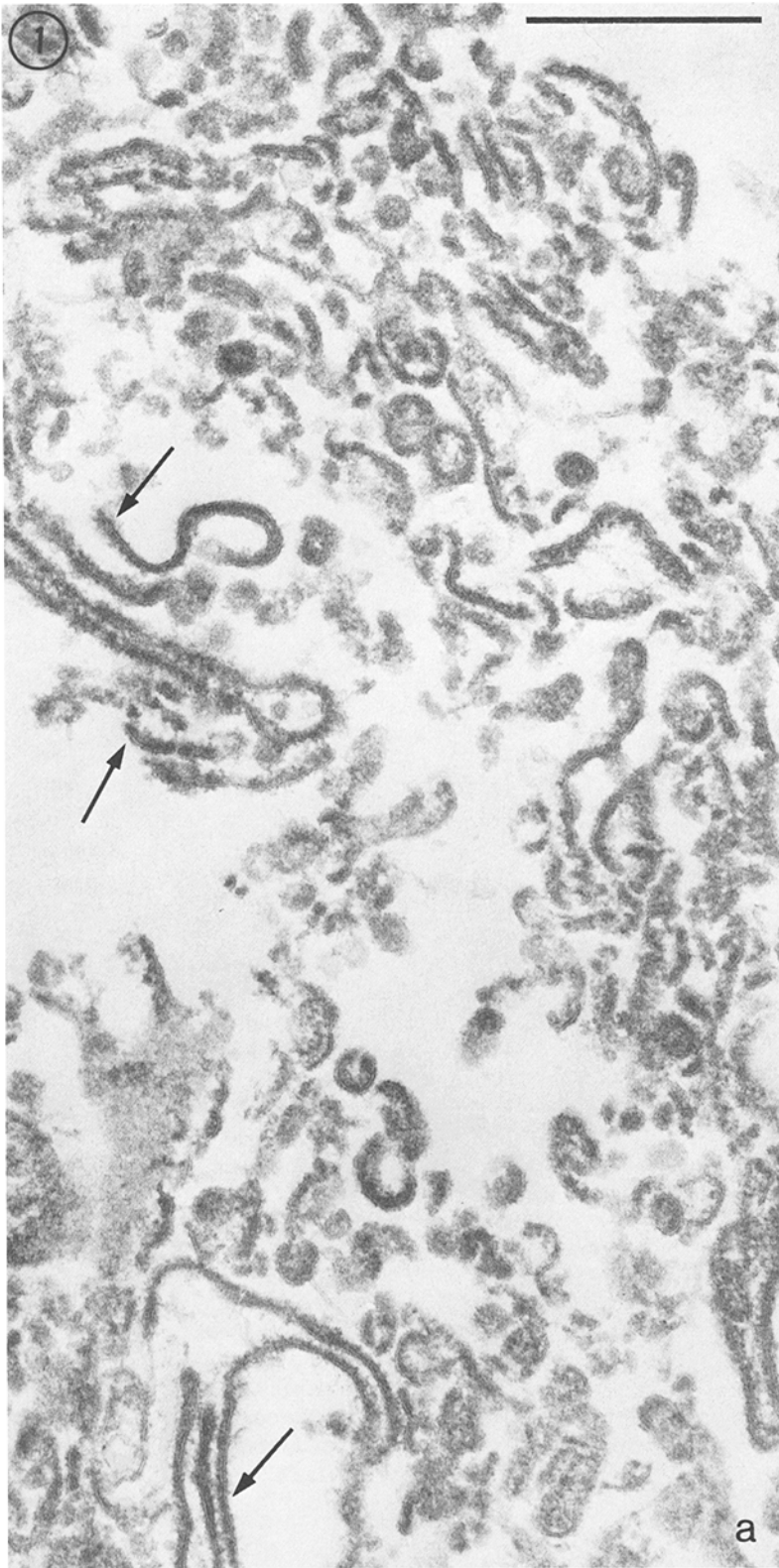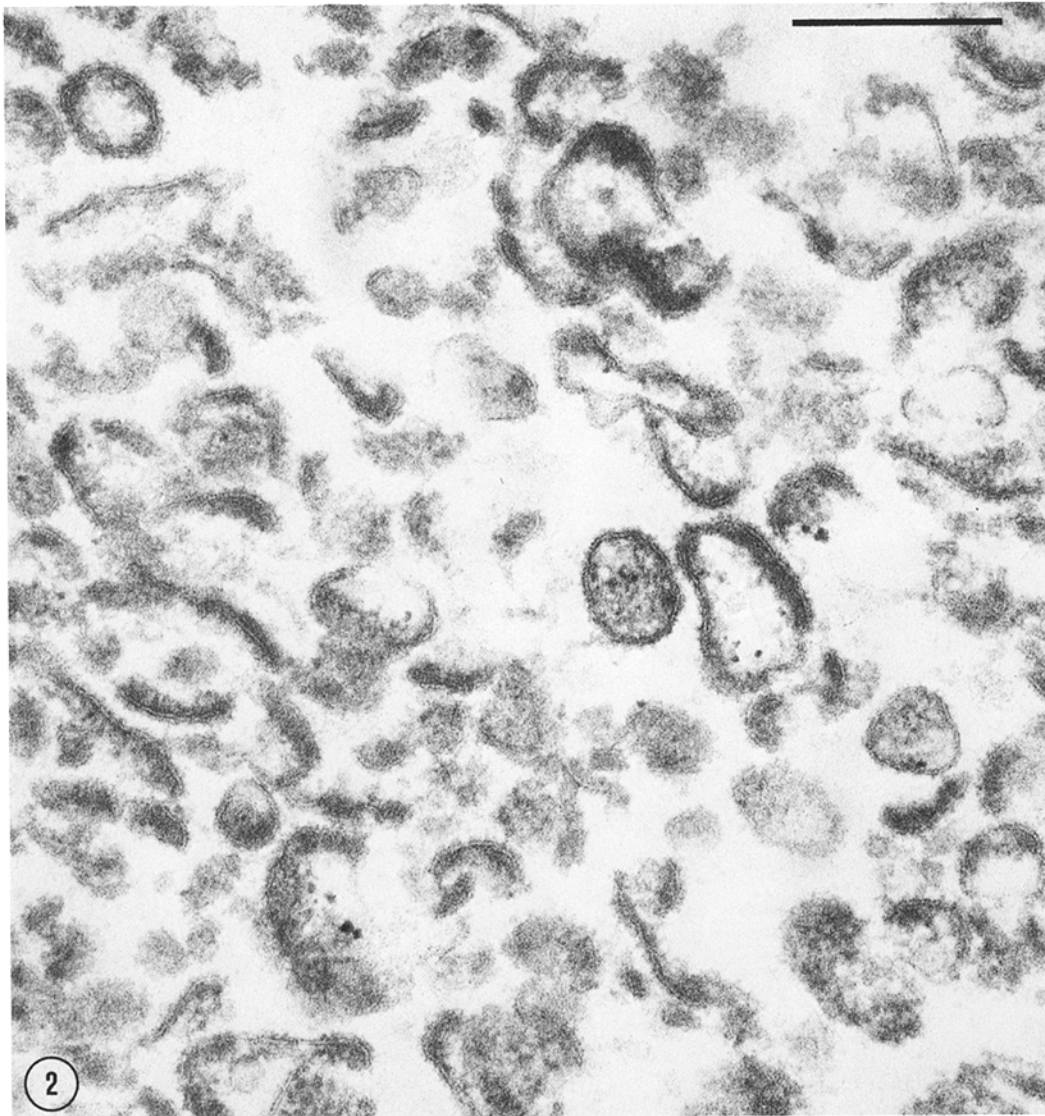
FIGURE 2 Survey micrograph of an MFGM fraction as it appears after more intensive disintegration, e.g. by sonication with and without high-salt treatment. Note the predominance of small membrane sheets in this fraction which, however, are still associated with the internal fuzzy layer. Bar, 0.2 μm. × 143,000.

RER vesicle fraction after sonication and treatment with high-salt concentrations is shown in Fig. 3b; ribosomes are released.

Biochemical Characterization of the Fractions

Data on the gross composition of bovine MFGM fractions differ widely in the various publications (for review, see reference 55) with respect to relative amounts of phospholipids, neutral lipids, and sterols. Such a diversity may be due to differences in the methods used to purify and wash the MFGM fractions. We have determined the amounts of lipids, phospholipids, cholesterol, protein, RNA, and DNA in bovine and human MFGM fractions before and after treatment with high-salt concentrations, and have compared them to the composition of intact bovine MFG and RER fractions (Table II).

The low contents of phospholipids and choles-

TABLE 1

Membrane Profile Lengths in MFG Fractions as Determined by Morphometry in Ultrathin Sections

Membrane component	Bovine MFG		Human MFG		Rat MFG	
	% Membrane profile length (% Weight protein*)					
(1) MFGM attached to MFG	33.0	(34.0)	47.3	(48.3)	55.1	(57.2)
(2) Total MFGM	82.0	(83.0)	65.9	(67.4)	56.8	(58.9)
(3) Plasma membranes attached to MFG	2.5	(2.6)	4.7	(4.7)	5.0	(5.2)
(4) Total plasma membranes	5.1	(5.3)	8.4	(8.6)	13.0	(13.5)
(5) Sum of (2) and (4) above	87.1	(88.3)	74.3	(76.0)	69.8	(72.4)
(6) RER	2.1	(1.7)	12.5	(11.7)	16.7	(14.7)
(7) Nuclear membranes	0.1	(0.1)	0.3	(0.3)	0.4	(0.4)
(8) Mitochondrial membranes	0.4	(0.3)	1.4	(1.3)	2.4	(2.1)
(9) Unidentified membranes	10.3	(9.6)	11.4	(10.7)	10.8	(10.4)
Total membranes	100	(100)	100	(100)	100	(100)
	3,900‡		2,500‡		1,500‡	

As to the criteria for the classification of the membranes, see reference 25.

* The figures in the parentheses indicate the approximate percentages of protein of the membrane components. The relative protein contents (% of total dry weight; cf. also Table II) used for the specific fractions are: bovine RER 63%, bovine MFGM 76%, human RER 65%, human MFGM 69%, rat RER 59%, rat MFGM 70%.

‡ Figures give values in micrometers of the total membrane profile length measured.

terol in MFGM fractions, relative to the higher levels in plasma membrane fractions from other cell types (e.g. references 7, 19, and 56), probably reflect the presence of the massive internal fuzzy coat (see above) and also explain the high buoyant density of MFGM (1.18–1.23 g/cm³). The molar ratio of cholesterol to phospholipid was 0.13 in the bovine RER fraction (0.22 in the corresponding fraction from rat) whereas it was 0.20 in the MFGM fraction from the cow (0.31 in rat MFGM). These figures for MFGM are markedly lower than those reported for plasma membranes isolated from this tissue (38) and from other tissues and cell systems (e.g. references 7, 16, 19, 24, and 56). MFGM fractions contained more neutral lipids relative to phospholipids than the microsomal membranes from the mammary gland, even after purification by sucrose gradient centrifugation (cf. also reference 55). After repeated washes and an extraction at high-salt concentrations, the phospholipid to neutral lipid ratio of the MFGM fractions increased considerably (see Table II). When the high-salt-extracted MFGM fraction was further treated with 0.1% deoxycholate (DOC, in 10 mM Tris-HCl buffer, pH 7.4), this ratio increased to values of more than 3, i.e. to values which are essentially identical with those of RER membranes (cf. Table II). In both MFGM and RER fractions from mammary gland as well as in RER from rat liver, this DOC treatment did not result in the extraction of signifi-

cant amounts of phospholipids. These observations indicate that the high contents of neutral lipids found in MFGM fractions by various authors (for review, see reference 55) are due to a loose attachment of these lipids, which are most probably derived from the fat droplet of MFG, to the MFGM.

DNA was not detected in MFGM fractions but was found at low but significant concentrations in total MFG. This DNA content can be attributed to the presence of occasional desquamated cells that are consistently detected in cream and MFG fractions by light and electron microscopy; such cells or cell fragments are not quantitatively recovered in the sediment of the centrifugation steps because of their fat droplet content and/or adsorption to MFG. The RNA to protein ratio is about twice as high in total MFG and MFGM from the rat as it is in the corresponding bovine fractions (Table II), in agreement with the higher frequency of RER elements in rat milk samples.

Cytochrome Content of the Fractions

Figure 4 shows difference spectra (samples treated with cyanide, ascorbate, NADH, or dithionite) at room temperature of purified, high-salt-extracted bovine MFGM fractions, which show the presence of a b-type cytochrome. With dithionite as the reducing agent, the predominant characteristic of the spectrum was a deep trough in the range of 450–470 nm, which is explained by a high
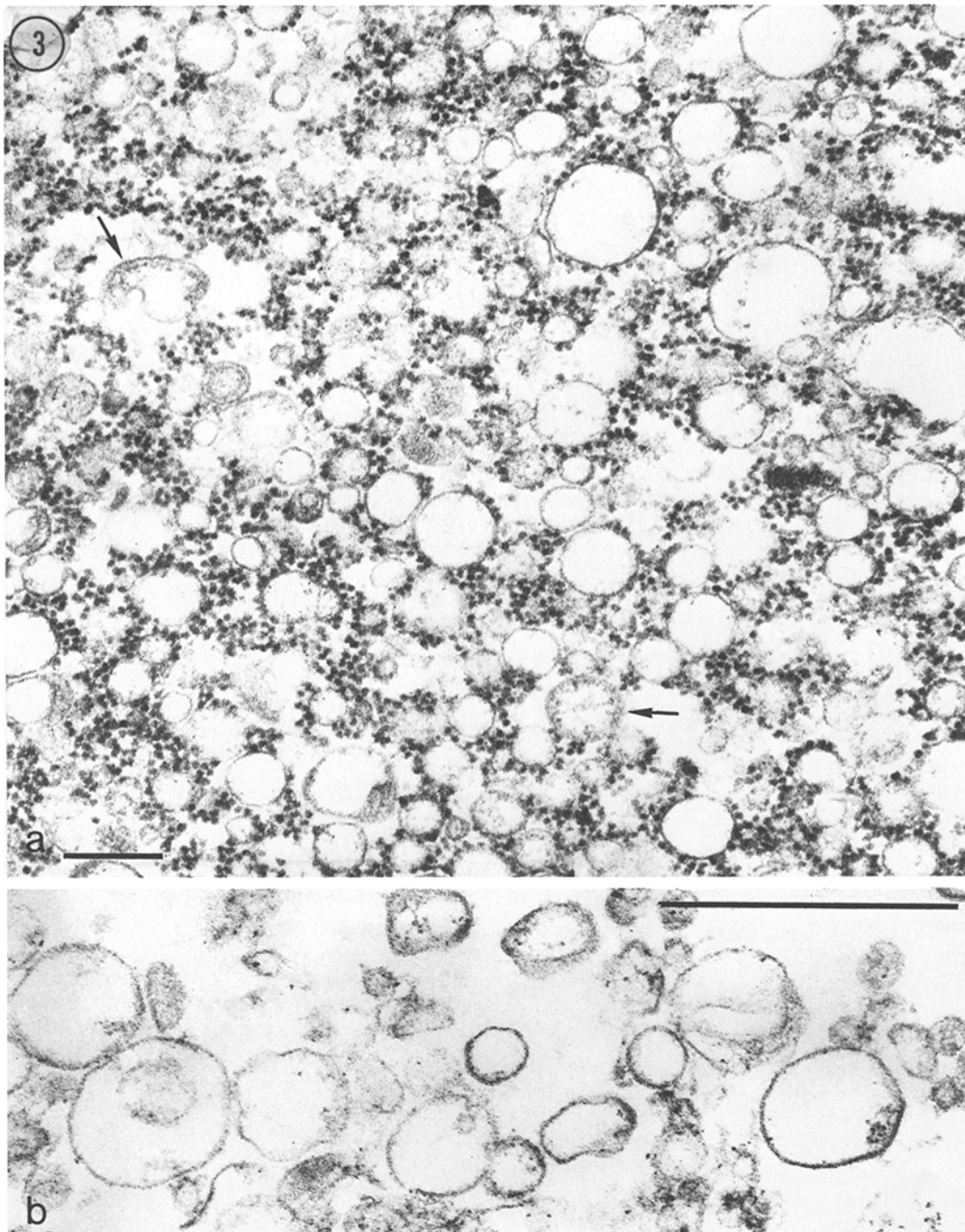
FIGURE 3a and b   Survey micrographs showing the purity of the rough microsomal fraction from lactating rat mammary tissue as it appears before (a) and after (b) treatment with very low- and high-salt concentrations. Note the abundance of membrane-attached ribosomes in Fig. 3a. The arrowheads in Fig. 3a denote two minor contaminants which apparently are plasma membrane-derived vesicles. Bars: (a) 0.2 and (b) 0.5 μm. (a) × 70,000; (b) × 84,000.

Table II

Characteristic Ratios (wt/wt and mol/mol) of Lipids, Protein, RNA, and DNA in Fractions from Milk and Mammary Gland

Fraction	Phospholipids / protein	Phospholipids / neutral lipids	Cholesterol / phospholipids	RNA / protein	DNA / protein
	wt/wt	wt/wt	(mol/mol)	wt/wt	wt/wt
Bovine					
RER	0.25	2.6	0.13	0.14	<0.002
RER, high salt extracted	0.35	3.2	0.14	0.05	<0.002
Total MFG	0.08	0.005	0.08	0.02	0.005
Purified MFGM	0.13	0.79	0.20	0.02	ND
Purified MFGM, extensively washed and high salt extracted	0.18	2.0	0.22	0.01	ND
Human					
Purified MFGM	0.22	1.3	0.18	0.03	ND
Purified MFGM, extensively washed and high salt extracted	0.27	2.2	0.18	0.01	ND
Rat					
RER	0.36	2.9	0.22	0.12	<0.002
RER, high salt extracted	0.45	3.6	0.24	0.04	<0.002
Crude MFGM	0.14		0.31	0.04	ND

ND means not detected.

concentration of flavins in the reduced state (see below). A hemoprotein component was indicated by peaks at about 427 and 562 nm. This hemoprotein was better recognized when the membrane fraction was treated with ascorbate instead of dithionite. Under these conditions, the flavins in the MFGM were not reduced, but the hemoprotein showed the typical reduced vs. oxidized difference spectrum of a b-type cytochrome, with peaks at 429, 531, and 562 nm. The reduction by ascorbate was a rather slow reaction but could be accelerated by the addition of catalytic amounts of N,N,N',N'-tetramethyl phenylenediamine. The ratio of the cytochrome concentration reduced by ascorbate relative to that reduced by dithionite was approx. 0.6-0.8, even under anaerobic conditions. Apart from the treatment with dithionite or ascorbate, the cytochrome was also partly reduced by either NADH (see Fig. 4) or NADPH (maximal reduction was achieved at about 0.3 mM with both substrates).

In the rough microsomes from lactating bovine mammary gland, a cytochrome with spectral properties similar to those of the cytochrome in the MFGM fraction was observed. This component was reduced by ascorbate, NADH, and NADPH, respectively; in the reduced vs. oxidized difference spectrum, peaks at 426, 530, and 560 nm were

found (see Fig. 5). The concentration of this pigment in the RER fraction exceeded that of the MFGM fraction by a factor of about seven.

In both MFGM and RER, the reduction of the cytochrome components by NADH was not affected by rotenone and antimycin. When the membranes were treated with cyanide (in the range of 0.3-2.0 mM final concentration), without the addition of any reducing agent, and the difference spectrum between this and an untreated reference was recorded, peaks that are typical for a reduced b-type cytochrome were obtained. Differences between the b-type cytochromes of MFGM and mammary RER existed in the exact positions of the Soret- and the α-band of the reduced components: 427 nm and 562 nm in the MFGM, compared to 426 nm and 560 nm in the RER membranes. With ascorbate as the reducing agent, the Soret-band in the MFGM fraction, but not in the RER fraction, was consistently located at 429 nm. In the difference spectra of the cyanide-treated fractions, the Soret-band was found at 428 nm in the MFGM and at 425 nm in the RER fractions (see Figs. 4 and 5). The cytochrome components of both membrane types resisted a solubilization by repeated low- and high-salt treatments (see Materials and Methods) as well as an extraction with 0.1% deoxycholate. The solubili-
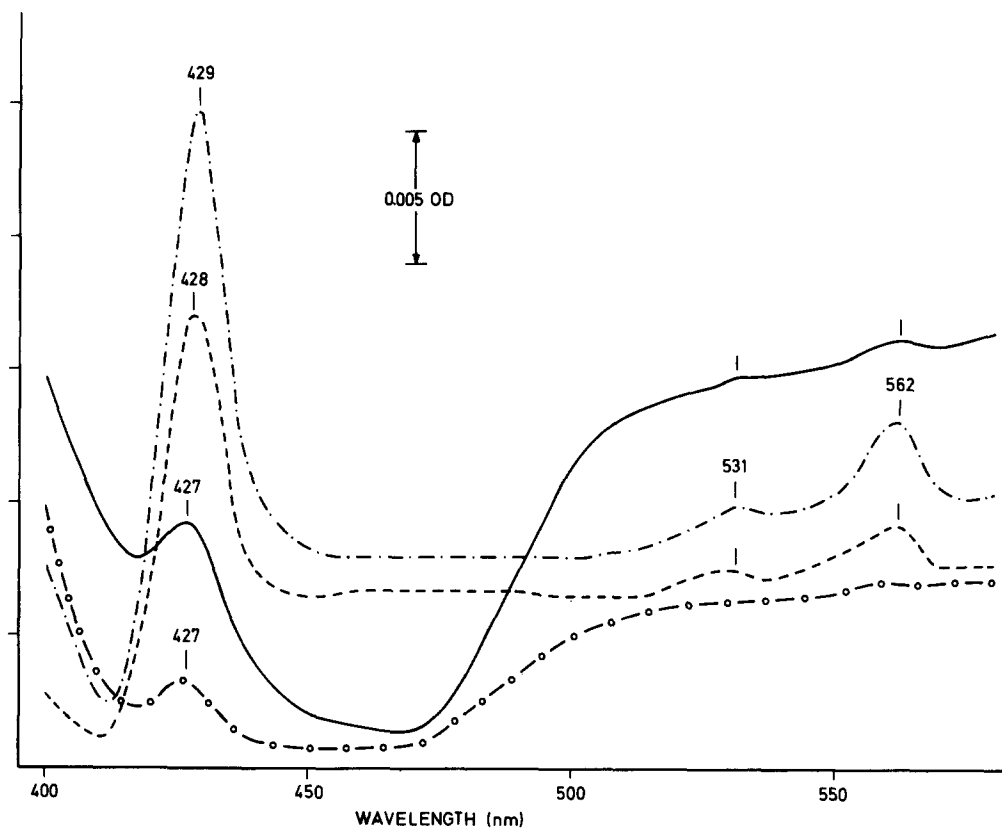
FIGURE 4 Difference spectra of purified bovine MFGM, high-salt extracted. Samples contained 1 mg (in the case of dithionite and NADH) or 10 mg (in the case of ascorbate and cyanide) protein in 1 ml 0.2 M potassium phosphate buffer. pH 7.4, and 50% glycerol. The base line was recorded from two identical samples under aerobic conditions at 20°C. The sample in one cuvette was then treated with 1 μmole KCN (– – – –), 5 μmoles ascorbate (– · – · –), or 0.1 μmole NADH (O–O–O), each dissolved in 10 μl H₂O, while the reference cuvette was supplemented with the corresponding volume of water. After thorough mixing, the difference spectrum between sample and reference was recorded. The difference spectrum between the dithionite-reduced sample and the untreated reference (————) was recorded after addition of a few grains of dithionite to the sample cuvette followed by a treatment with a gentle stream of nitrogen for 5 min. The curves shown are corrected for the base line.

zation of the b-type cytochromes by increasing concentrations of Triton X-100 (0.5–5 mg Triton X-100 per mg protein; see Materials and Methods) differed in both fractions, MFGM and RER. Even at very high detergent concentrations, only about 23% of the cytochrome and 33% of the total membrane protein were solubilized from the MFGM, whereas for the microsomal membranes these figures were 71% and 69%, respectively.

When the cytochrome spectra of MFGM and mammary RER were compared to those of mammalian liver RER, marked similarities were noted. Like the mammary b-type cytochromes, the well-characterized microsomal cytochrome b_5 from mammalian liver (cf. references 31, 32, and 68) is

reducible by ascorbate, NADH, and NADPH, and its reduction by NADH is insensitive to rotenone and antimycin. In both liver and mammary microsomes, the Soret-bands of the b-type cytochromes (reduced by ascorbate or dithionite) are at identical positions (see Fig. 5, inset). However, some differences between the two systems were observed. When liver microsomes were treated with cyanide, a broad maximum at about 445 nm and a corresponding minimum at 405 nm were recorded, but no distinct peaks as for MFGM and mammary RER (cf. reference 14). In addition, the α-band of the reduced hepatic cytochrome b_5 is asymmetric with a maximum at 555 nm and a shoulder at 560 nm. In low temperature difference
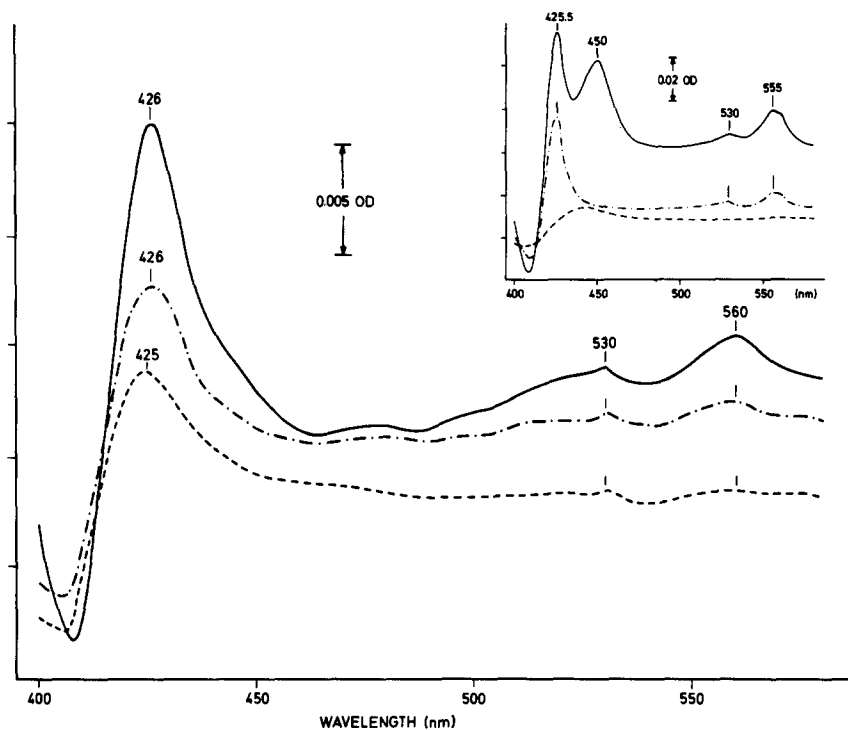
FIGURE 5 Difference spectra of purified high-salt-extracted RER fractions from bovine mammary gland. The sample in one cuvette was first treated with 1 μmole KCN (- - - -), then with 5 μmoles ascorbate (- · - · -), and finally with a few grains of dithionite (———). Protein concentration was 1 mg/ml. Other conditions were as in Fig. 4. The *inset* shows, for comparison, difference spectra from rat liver RER membranes. The fraction was treated with KCN (- - - -), ascorbate (- · - · -), and dithionite (———) in the same way as the mammary microsomes. Protein concentration was 1 mg/ml. Under these conditions, the α-band of cytochrome b_5 at 555 nm appears asymmetrical and cytochrome P-450 is clearly visualized by its peak at 450 nm. With mammary RER, the α-band is a symmetric peak at 560 nm, and no cytochrome P-450 is detectable. Note the completely different cyanide spectra of the liver and mammary RER fractions.

spectra, this band is split into two distinct peaks (e.g. reference 30). The α-band of the b-type cytochromes from MFGM and mammary RER appeared as one symmetrical peak at about 560 nm that was not split at low temperatures (cf. Figs. 4 and 5).

Cytochrome P-450 which is present in high amounts in liver ER (23, 37; see also *inset* of Fig. 5) could not be detected in MFGM, the limit of detection in our carbon monoxide (CO) difference spectra being approx. 2.5 pmoles/mg protein. In purified RER membrane fractions, a small shoulder in the range of 440–460 nm was occasionally found in the CO-spectra which, however, could not clearly be attributed to cytochrome P-450. In any case, the amount of cytochrome P-450 that might be present in this or in any other endomembrane fraction from the mammary gland would be

less than 10 pmoles/mg protein, a value that is much lower than that of the corresponding fractions from liver (less than 5%). Further, using codeine as a substrate, we were unable to detect any trace of N-demethylase activity, an activity which in liver ER is dependent on the presence of cytochrome P-450 (2), in MFGM or mammary cell fractions.

Liver cytochrome b_5 is readily solubilized by detergents such as Triton X-100 (52). As described above, the b-type cytochrome from MFGM was almost completely resistant to Triton X-100. In the mammary RER fractions, however, most of the cytochrome could be solubilized under such conditions.

The cytochrome components in the MFGM and RER fractions from the mammary gland were not confined to the cow but were also found in the

corresponding fractions from the women and from rat, mouse, and goat. The concentrations of these pigments, however, differed among the various species (see Table III). Human and murine MFGM fractions were especially rich in cytochrome. The cytochrome concentration in these membrane fractions was in the range of that in the corresponding mammary RER membranes.

The insensitivity to rotenone and antimycin of the NADH-dependent reduction of the b-type cytochromes in the MFGM and RER fractions distinguishes these cytochromes from the mitochondrial cytochrome b. In all MFGM fractions studied, mitochondrial contamination could be ruled out. The characteristic peaks of the cytochromes c + c_1 and aa_3 were not detected in reduced vs. oxidized difference spectra recorded at either room temperature or low temperature. Activities of the mitochondrial enzymes cytochrome c oxidase, rotenone-sensitive NADH oxidase, and succinate dehydrogenase were also absent from the MFGM fractions. Carbon monoxide-binding hemoproteins such as cytochrome P-450, cytochrome a_3, hemoglobin, and peroxidase were not found spectrophotometrically. In addition, the activity of lactoperoxidase, a characteristic milk serum (milk minus the fat globules) protein (59), was not detected in MFGM. Purified MFGM fractions were essentially devoid of caseins, as evidenced by the very low content of protein-bound phosphorus and the absence of constituents migrating with caseins on gel electrophoresis.[1] In addition, casein micelles (cf. reference 26) were not observed on morphological examination. The maximally possible contamination of MFGM fractions by ER and other membrane components was best determined by the morphometric analysis as described above. It is evident from these data that the low contamination of less than 3% membranes present in total bovine MFG fractions cannot explain the high content of the b-type cytochrome in MFGM. This cytochrome in bovine MFGM cannot be derived from the milk serum since this pigment is not found in the soluble fractions from cow's milk.

In purified RER fractions from the mammary gland, traces of cytochrome aa_3 were occasionally detected by a small shoulder at 445 nm in the dithionite-reduced difference spectra (cf. Fig. 5). From the cytochrome aa_3 content of crude mito-

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[1] Keenan, T. W., C. Freudenstein, and W. W. Franke. Manuscript in preparation.

Table III

Contents of b-Type Cytochromes in MFGM and Mammary RER

	High salt treatment	b-Type cytochrome in MFGM	b-Type cytochrome in RER
		nmol/mg protein*	nmol/mg protein*
Cow	−	0.030	0.224
	+	0.045	0.326
Goat	−	0.051	
	+	0.109	
Woman	−	0.154	
	+	0.157	0.095
Rat	−	0.041	0.110
	+		0.160
Mouse	−	0.145	0.130
	+		0.142

In human MFGM fractions, no enrichment in the cytochrome content was observed after the treatment at high ionic strength. This correlates with the finding that here, in contrast to the other species, a considerable part of the cytochrome was detected in the high salt supernate.
* Assuming a molar extinction coefficient of 160 cm^{-1} mM^{-1} (for reference, see 36).

chondrial fractions from the same tissue, a contamination by inner mitochondrial membranes in the RER fractions of less than 5% was estimated. Cytochrome oxidase and NADH oxidase activities in these fractions were even lower, compared to those of the mitochondrial fraction. Succinate dehydrogenase activity and the cytochromes c + c_1 were not detected in purified RER fractions. These results correspond well with the data obtained by morphometry (see above). In fractions that had been washed with phosphate buffer (see Materials and Methods), no contaminating hemoglobin was detected by spectral analysis.

Table IV shows the distribution of protein, phospholipids, and the b-type cytochrome in subfractions from bovine MFGM. When the MFGM fraction was treated at high ionic strength, about 20% of the total protein was extracted into the supernate whereas all the phospholipids and cytochromes were recovered (within the experimental limits) in the residual membrane pellet. These data indicate that the cytochrome is not likely to be an ionically membrane-attached protein but rather is probably an intrinsic component of the membrane. Consequently, for comparison with other membranes, the cytochrome concentration of MFGM is better expressed on a phospholipid than on a protein basis (see Table IV).

Flavin Content and Some Redox Enzyme Activities of the Fractions

So far, the only flavoprotein known to occur in bovine MFGM is xanthine oxidase (4, 9, 17). As can be seen from Table V, both the flavin content and the xanthine oxidase activity of bovine MFGM fractions were high. Some evidence in favor of an identity of the flavoprotein of bovine MFGM with xanthine oxidase is provided by the findings that the MFGM flavin was reducible by NADH but not by NADPH (see above and Fig. 4) and that it was also reducible by hypoxanthine (5 mM final concentration) under strictly anaerobic conditions. A similar ratio of xanthine oxidase activity to flavin content was observed in both bovine and rat MFGM. Despite their high flavin concentrations, human MFGM fractions displayed only low xanthine oxidase activities, even when the membranes were prepared (in this case within 90 min) from freshly collected milk samples. The specific activity of xanthine oxidase in bovine MFGM increased by about 50% in the sHSE and decreased in the high-salt-extracted membranes. About 70% of the total flavin content in MFGM

was recovered in the membrane fraction after a high-salt treatment. There was no correlation between flavin content and xanthine oxidase activity in the various subfractions from MFGM, which might be explained by the observation that the enzyme exists in inactivated forms (see reference 8). In mammary gland, all fractions had only very low xanthine oxidase activities, compared to the MFGM fractions. The highest specific activities were observed in total RER membranes (see Table V).

The microsomal electron-transport system of mammalian liver contains both NADH- and NADPH-cytochrome c reductases (see references 33, 60, and 67). We measured these enzyme activities in our membrane fractions from mammary gland and milk and found significant though relatively low values compared to liver material (Table V). In bovine MFGM, the NADH-cytochrome c reductase activity was only about 3% of that present in the corresponding RER fraction. Since we found that this enzyme was rapidly inactivated upon prolonged storage (more than 80% inactivation within 24 h at 4°C; see also reference 17), the very low activity in cow's milk might be

TABLE IV
Distribution of Protein, Phospholipids, and b-Type Cytochrome in Subfractions from Bovine MFGM

Fraction	Protein content	Phospholipid content	b-Type cytochrome		
	%	%	pmol/mg protein	pmol/mg phospholipid	Total content %
Purified MFGM	100	100	33	254	100
Purified MFGM, high salt extracted	73.1	101	41	228	91
isHSE of MFGM	13.8	<3	<4	–	<1.5
sHSE of MFGM	5.8	0	0	–	0
Total recovery	92.7	101–104			91.0–92.5

TABLE V
Flavin Content and Enzyme Activities in MFGM and Mammary RER

Fraction	Flavin content	Xanthine oxidase*	Rotenone-insensitive NADH-cytochrome c reductase‡	NADPH-cytochrome c reductase‡
	nmol/mg protein			
Bovine RER	<0.04	2.5	105	4.5
Bovine MFGM	0.43	180	4.2	1.6
Human MFGM	0.51	7.6	7.3	1.2
Rat RER	<0.02	2.0	22.2	5.3
Rat MFGM	0.04	10.4	8.3	5.0

* Specific activity is expressed as nanomoles uric acid formed per minute per milligram protein.
‡ Specific activities are expressed as nanomoles cytochrome c reduced per minute per milligram protein.

explained by the relatively long time between milk fat secretion and milking. In rat MFGM, NADH-cytochrome c reductase activity was 38% of that of rat RER. This high value cannot be explained by contamination. High-salt extractions of the various fractions resulted in an increase of the specific NADH-cytochrome c reductase activity in the membrane material, indicating that this enzyme is an intrinsic membrane protein.

NADPH-cytochrome c reductase activity was detected in low but significant levels in all of the membrane fractions studied. The activity measured in MFGM fractions was only slightly below that found in the corresponding RER fractions. This NADPH-cytochrome c reductase activity was rather stable on storage and experimental stresses. In the bovine MFGM, this activity was partially extracted by high salt, in contrast to the NADH-dependent enzyme.

In liver microsomes, cytochrome b_5 transfers electrons from NADH and, to a lesser degree, from NADPH to a fatty acyl coenzyme A desaturase (51, 69). Since fatty acyl coenzyme A desaturase activity had been measured in microsomes from lactating ruminant (6, 46) and mouse (62) mammary glands and in fresh goat's milk (45), we examined the possible involvement of our b-type cytochrome in desaturase reactions. We confirmed the presence of this activity in RER fractions from cow and mouse mammary glands but failed to detect it in rat RER and in MFGM fractions from all the species studied. These negative results were obtained with NADH and/or NADPH as electron donors, with palmityl-CoA or stearyl-CoA as substrates, and with or without glycerol-3-phosphate as an acyl acceptor. Negative results were also obtained with freshly secreted milk and MFGM derived therefrom (cows were milked three times at hourly intervals, and only the last milk sample was used).

Reduction and reoxidation of the NADH- and NADPH-reduced b-type cytochrome were recorded with a dual wavelength spectrophotometer at the wavelength pair 427 and 418 nm (Fig. 6). In RER fractions, rapid reoxidation was observed that was dependent on the presence of oxygen and was not inhibited by 0.3 nM cyanide. A NADH- and NADPH-dependent oxygen consumption which is related to the reoxidation of the b-type cytochrome was also observed with RER fractions (Fig. 6). In MFGM fractions, reduction and reoxidation of the cytochrome by NADH could not be measured with the dual wavelength technique due
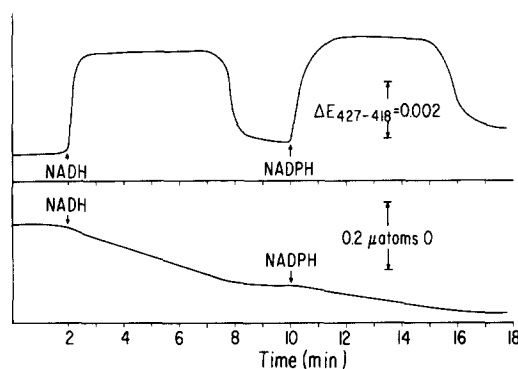

FIGURE 6 Reduction and reoxidation of mammary b-type cytochrome and oxygen consumption produced by NADH and NADPH in purified RER fractions from rat mammary gland. 2.0 ml of RER membranes in 0.3 M sucrose-TKM (5.8 mg protein/ml) were incubated at 23°C. At the time indicated (arrows), 50 nmoles NADH and NADPH, respectively, were added. b-Type cytochrome reduction and reoxidation was measured by the change in the absorbance difference between the peak of the reduced form (427 nm) and the isosbestic point (418 nm). Oxygen consumption was analyzed polarographically.

to the spectral interference of the flavin. Since no spectral change was observed in the NADH-reduced vs. oxidized difference spectrum over 2 h, we conclude that the cytochrome in MFGM is not reoxidizable. With NADPH, the reduction of the cytochrome in MFGM was too low for quantitative analysis. We found a small but steady antimycin- and rotenone-insensitive NADH- (but no NADPH-) dependent oxygen consumption (ca. 5 natoms oxygen consumed/min/mg protein) in purified bovine MFGM. This oxygen uptake decreased by about 50% during high-salt treatment and was slowly and irreversibly abolished by increasing concentrations of cyanide (0.5-5 mM).

DISCUSSION

In view of the current concept of membrane diversity and mechanisms of membrane biosynthesis, the reported compositional differences between plasma membranes and endomembranes, particularly those of the ER system are of special importance (for references, see 24, 47, and 53). Most of the procedures for isolating plasma membranes from glands and epithelia are based on the original method of Neville (50; for review, see reference 71). These methods tend to select and to enrich for lateral plasma membrane components, such as junctional complexes, and result in large losses of

apical membrane components. The MFGM fraction, however, provides isolated components derived exclusively from the cell apex, i.e., the surface region with the apparently highest turnover as suggested from morphological observations (for references see 26). Therefore, we have undertaken a study on the composition of the MFGM, in comparison with the RER.

Cytochrome b_5 and NADH- and NADPH-cytochrome c reductase are well known constituents of the ER in many eucaryotic cells (for reviews, see references 15 and 64) and have also been reported as constituents of other elements of the endomembrane system such as the nuclear envelope (for references, see 23 and 37), the outer mitochondrial membrane (e.g. references 27 and 65), and the dictyosomal membranes (e.g. references 21 and 48). The occurrence of such redox components in surface membranes of eucaryotic cells has remained obscure (for negative statements, see references 21 and 35). Vassiletz et al. (70) mentioned the presence of cytochrome b_5 in plasma membrane fractions from rat liver; however, the reported concentration, on a protein basis, was only 9% of that present in the corresponding microsomal fraction, a value that is too low to be considered significant without a careful examination and determination of RER contaminants. In plasma membrane fractions from KB cells, Charalampous et al. (12) reported cytochrome b_5 contents and NADPH-cytochrome c reductase activities almost twice as high as those measured in their "microsomal" fraction; however, it is questionable whether the 100,000-g pellet from homogenates of cultured carcinoma cells really represents RER elements. The presence of significant amounts of NADH-cytochrome c reductase activity in isolated plasma membranes has been a subject of debate (e.g. references 12, 18, 20, 28, and 70). Very low NADH-cytochrome c reductase activities have been reported already in crude MFGM preparations (17). The present study shows that a b-type cytochrome is a constituent of the MFGM and thus suggests that this component is also present in the apical plasma membrane of the lactating mammary cell. This cytochrome is not confined to bovine MFGM but is also found, in some cases at much higher concentrations, in MFGM from other species. A similar b-type cytochrome is present in the RER of lactating mammary glands. The cytochromes in these two membrane systems are distinct from the mitochondrial cytochrome b but have properties in common with the cytochrome

b_5 of liver microsomes. They occur in the ER, they are reducible by ascorbate and by NADH and NADPH, and the latter two reactions are mediated by an antimycin- and rotenone-insensitive NADH-cytochrome b_5 reductase and a NADPH-cytochrome b_5 reductase, respectively (see Fig. 6). Like the liver cytochrome b_5, the cytochrome of the mammary RER is readily reoxidized by molecular oxygen. There are, however, differences between the b-type cytochromes from MFGM and mammary RER and cytochrome b_5 from the liver. The peaks in the reduced vs. oxidized difference spectra are located at longer wavelengths than in liver microsomes, the α-band of the reduced pigments does not split into two peaks at low temperatures (for liver cytochrome b_5, see reference 30), and the oxidized pigments when treated with cyanide show the characteristic difference spectra of a reduced b-type cytochrome. In liver microsomes, only cytochrome P-450 reveals a cyanide difference spectrum (14) which is completely different from that observed in MFGM and mammary microsomes (Fig. 5). Cytochrome P-450 is not found in MFGM and mammary RER.

The cytochromes in both membrane systems, bovine MFGM and RER, are recovered in the high-salt-extracted membranes, indicating that these pigments are intrinsic membrane-bound proteins. When the b-type cytochrome concentrations of bovine MFGM and RER fractions are calculated per weight phospholipid, the content of the MFGM is almost 30% of that of the RER. This value is far too high to be explained by contamination of MFGM fractions with ER vesicles entrapped during milk fat globule budding (see the morphometry data of Table I). When protein amounts of the b-type cytochrome in MFGM and RER fractions from bovine milk and mammary tissue are calculated, assuming a mol wt of 16,700 as for cytochrome b_5 (references in 52 and 68), it is found that this pigment represents only about 0.1% (in MFGM) and 0.6% (in RER) of total protein of the membrane fractions. When a similar estimation is made for the flavoprotein components, much higher values (6-9% of total MFGM protein) are obtained (cf. also reference 9). In human MFGM, the b-type cytochrome concentration is almost as high as the cytochrome b_5 content of mammalian liver microsomes, when expressed on a phospholipid basis (for comparison, see references 15, 21, 23, 35, and 37).

The cytochromes in the RER and in the MFGM differ in three properties. (a) The ascorbate-re-

duced pigment of the RER has a spectral peak at 426 nm, whereas in MFGM this peak is located at 429 nm. (*b*) The cytochrome present in RER is rapidly reoxidized by molecular oxygen, whereas the MFGM cytochrome is not. (*c*) The RER-cytochrome is solubilized with Triton X-100, whereas the MFGM cytochrome is resistant to detergent solubilization. We cannot decide whether the mammary microsomal cytochrome and the MFGM cytochrome are identical. The differences reported need not reflect different molecular species but may reflect differential influences in the specific membrane compartment.

Enzyme activities that are associated with cytochrome b_5 in liver are found in MFGM and mammary RER as well. Rotenone-insensitive NADH-cytochrome *c* reductase activities determined in MFGM are clearly below the activities found in the corresponding RER fractions. Since this activity is very labile to storage in both membrane fractions (see also reference 17 and 19), the low values for bovine MFGM may be explained by a postsecretory inactivation of the enzyme in the acini and ducts of the gland. In the rat, the enzyme activity in MFGM is almost 40% of that observed in rat mammary RER, indicating that it is not due to contamination by ER-derived elements (see Table I). NADPH-cytochrome *c* reductase activity, which is generally low relative to the activities of liver fractions (23, 35, 37), is almost as high in the MFGM as in the RER fractions (see, however, reference 58). In contrast to the NADH-dependent enzyme activity, it is relatively stable towards storage but is readily extracted by high-salt concentrations. Both NADH (in the presence of rotenone or antimycin) and NADPH reduce the *b*-type cytochrome of MFGM and RER in an enzymatic reaction (Fig. 6) that is probably related to the cytochrome *c* reductase activities mentioned above. In the RER, the reoxidation of the NADH- and NADPH-reduced cytochrome was accompanied by oxygen consumption. These "NADH oxidase" and "NADPH oxidase" activities were distinguished from mitochondrial respiratory components by their insensitivity to inhibitors of the mitochondrial respiratory chain. The low NADH-dependent oxygen consumption in MFGM is not associated with a reoxidation of the cytochrome. This MFGM NADH oxidase activity, which can be distinguished from the NADH oxidase activities of the ER and the mitochondria by its different response to cyanide, is probably due to the membrane-bound xanthine oxidase (see

references 9 and 41). The very high flavin concentration in bovine MFGM and the high xanthine oxidase activity distinguish this fraction from all eucaryotic surface membranes studied to date.

We do not know the biological function of these redox constituents in the MFGM and in the apical plasma membrane, and we do not know the mode and the route of the incorporation of such surface membrane components. From the mechanism of milk fat secretion, it is obvious that the mammary epithelial cell must provide a considerable net production of these proteins in order to compensate for the losses of apical surface with the fat globules (for detailed discussions, see references 26 and 55). A simple concept would be that these proteins are contained in the membranes of the casein secretory vesicles that are known to fuse with the apical surface membrane (26).

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