B-TYPE CYTOCHROMES IN PLASMA MEMBRANES ISOLATED FROM RAT LIVER, IN COMPARISON WITH THOSE OF ENDOMEMBRANES

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ABSTRACT

Fractions of plasma membranes, Golgi apparatus, endoplasmic reticulum (ER), and nuclear envelope were isolated from rat liver and were characterized by electron microscope and biochemical methods. The purity of the fractions was controlled by morphometry and by marker enzyme activities. Amounts of cytochromes b_5, P-450, and P-420 were measured, as well as the NADH- and NADPH-cytochrome *c* reductase activities. The pigments of the microsomal electron transport system were found in all membrane fractions in relatively high amounts, thus excluding an origin by microsomal contamination. Purified preparations of plasma membrane and Golgi apparatus contained ~30% of the cytochrome b_5 and cytochrome P-450 + P-420 found in ER membranes. Plasma membranes were also characterized by a high ratio of P-420/450. Degradation of cytochromes P-450 and P-420 was relatively rapid in all fractions, except in the ER. Cytochrome b_5 extracted from plasma membranes was spectrophotometrically and enzymatically indistinguishable from ER cytochrome b_5. However, immunological characterization with rabbit antibodies against the trypsin-resistant core of microsomal cytochrome b_5 showed the presence of at least two types of cytochrome b_5 in ER membranes, in contrast to the plasma membranes in which only one of these components was detected. This immunological differentiation also demonstrates that the plasma membrane-bound cytochrome b_5 is endogenous to this membrane and does not reflect contamination by ER elements.

We conclude that cytochromes b_5, P-450, and P-420 are not confined only to ER and nuclear membranes but also occur in significant amounts in Golgi apparatus and plasma membranes. The findings are discussed in relation to observations of similar redox components in Golgi apparatus, secretory vesicles, and plasma membranes of other cells.

KEY WORDS cytochromes · endomembranes · plasma membranes · redox enzymes · rat liver

The endoplasmic reticulum (ER) of mammalian hepatocytes contains two classes of b-type cytochromes (50, 62): (*a*) cytochrome b_5, which transfers electrons from a flavoprotein (NADH-cytochrome b_5 reductase) to a stearyl coenzyme A

desaturase and probably to other electron acceptors as well (e.g., references 25, 42, and 53); and (*b*) several closely related species of the carbon-monoxide-binding pigment, cytochrome P-450, which accepts electrons from a flavin-containing NADPH-cytochrome *c* reductase for a large variety of hydroxylation and dealkylation reactions (e.g., references 12, 52, and 63). A second group of carbon-monoxide-binding pigments found in the ER, designated cytochrome P-420, is widely considered a degradation product of cytochrome P-450 (e.g., references 30 and 50). Evidence exists that in liver ER these two electron transport systems are interconnected (25, 42). Cytochrome b_5 and cytochrome P-450 have been also described in the ER of many other tissues and cells (for reviews, see references 12, 23, and 40). In addition to its presence in ER membranes, cytochrome b_5 is also known to occur in nuclear membranes (1, 17, 36), in outer mitochondrial membranes (15, 59), and in membranes of the Golgi apparatus (2, 3, 14, 29, 47; see, however, reference 15). Data on the occurrence of cytochrome P-450 in these membranes are conflicting. This pigment has been reported present in hepatic membranes of the rat and the rabbit (17, 28) but absent in nuclear membranes from bovine liver (1; for review see references 16 and 36). It has been found in Golgi membrane preparations from mammalian liver (29, 47; see, however, references 2, 14, and 21). Cytochrome P-450 has recently been detected in outer mitochondrial membranes (64; see, however, reference 59). By contrast, various authors have reported that cytochromes do not occur in liver plasma membranes (10, 14, 15, 21, 29; see, however, reference 65).

In previous articles, we have shown that a derivative of the apical plasma membrane of the lactating mammary gland cell, the milk fat globule membrane, contains both b-type cytochromes b_5 and P-420 (4, 31). In the present study we demonstrate that plasma membranes of rat hepatocytes also contain cytochromes of the b_5 and P-450 types. Compared to those present in the ER, the corresponding electron transport systems in plasma membranes, in Golgi apparatus, and in nuclear membranes are characterized by some distinct features.

MATERIALS AND METHODS

Animals

2-mo-old Sprague-Dawley rats (150–250 g body wt) that had been fasted for 15 h were anaesthetized with ether, and the livers were perfused with 50–100 ml of physiological saline solution before removal of the organ. Essentially the same results were obtained with female and male rats.

Isolation Procedures

The livers were washed in physiological saline, cut into small pieces, squeezed through a fine-meshed metal sieve to remove strands of connective tissue, and the minced tissue was transferred into two vol of cold (0°–4°C) homogenization medium (0.5 M sucrose, 1% dextran, 5 mM $MgCl_2$, 40 mM Tris-maleate buffer, pH 6.5; cf. reference 46). After homogenization with a loosely fitting Potter-Elvehjem device (five strokes slowly up and down at 150 rpm and 4°C), the material was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 1,500 *g* for 15 min. The supernate was diluted with 40 mM Tris-maleate buffer (pH 6.5) which contained 5 mM $MgCl_2$ to a 0.35 M sucrose concentration, and mitochondria were sedimented by two centrifugation steps at 10,000 *g* for 10 min. The "total ER" fraction was pelleted from this supernate by a 100,000 *g* spin for 1 h. Rough and smooth ER fractions were resuspended in 1.0 M sucrose (5 mM $MgCl_2$, 40 mM Tris-maleate buffer, pH 6.5) and were separated on a discontinuous sucrose gradient (layers containing 1.8, 1.4, 1.17, and 1.0 M sucrose, same buffer). The smooth ER banded at the 1.0/1.17 M sucrose interface and most of the rough ER at the 1.2/1.4 M sucrose interface.

The fluffy upper part of the pellet from the first 1,500 *g* centrifugation was carefully removed and used for isolation of Golgi apparatus as described (46). The tightly packed bottom portion of the pellet was resuspended in 0.4 M sucrose which contained 70 mM KCl, 2% gum arabic, and 10 mM Tris-HCl, pH 7.4, and was further processed for the isolation of nuclei and nuclear membranes as previously described (17, 20). Plasma membranes were isolated in sodium bicarbonate buffer as described (20). Usually, all fractions were sonicated (see reference 17) and extracted with 1.5 M KCl (10 mM Tris-HCl buffer, pH 7.4) in the cold (0°–4°C) for 1 h and were collected as described (20).

Specific detergent treatments used are mentioned under Results. To minimize oxidation of sulfhydryl groups or proteolysis, some isolation experiments were done with media which contained 2 mM mercaptoethanol or 0.1 mM phenyl methane sulfonylfluoride. Alternatively, plasma membranes were also isolated in Tris-maleate buffer, pH 6.5, and nuclear membrane and ER fractions were prepared by using media buffered with either sodium bicarbonate, pH 8.0, or Tris-HCl, pH 7.4, with or without $CaCl_2$ or $MgCl_2$.

Before spectrophotometry, membrane fractions were resuspended in 0.1 M sodium pyrophosphate buffer, pH 7.4, and resedimented at 100,000 *g* for 90 min to remove traces of hemoglobin. The fractions were finally resuspended in 0.3 M sucrose which contained 70 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris-HCl buffer, pH 7.4,

to a protein concentration of ~5 mg/ml, and were analyzed directly. Usually, preparations of ER and Golgi apparatus membranes were analyzed 3 h after sacrifice of the animals, whereas preparations of nuclear and plasma membranes were analyzed ~5 h after sacrifice.

Chemical Determinations and Enzyme Assays

Protein was measured by the method of Lowry et al. (41) or with the biuret reaction (22). RNA was extracted and determined by the method of Schmidt and Thannhauser (57). Sialic acids were determined as described (20). Lipids were extracted, and lipid phosphorus was determined as described (37). NADH- and NADPH-cytochrome *c* reductase activities were determined at various protein concentrations (32). Cytochromes were analyzed at −196° and 20°C (31, 32). In fractions with high contents of cytochrome P-420, the absorbance of the Soret band of the dithionite difference spectra could not be used for the determination of cytochrome b_5 (see reference 4). Therefore, cytochrome b_5 content was estimated from the absorbance at 556 nm at 20°C (62). Cytochrome P-450 and P-420 were determined as described by Omura and Sato (50); when both these cytochromes occurred in high amounts, a correction for the overlap of their absorbances was necessary (see reference 30). Succinate dehydrogenase and cytochrome *c* oxidase were measured polarographically and spectrophotometrically (32, 33). 5′-Nucleotidase activity was measured with 5′-AMP as substrate (24) and glucose-6-phosphatase by a micromodification of the assay described by Nordlie and Arion (49). UDP-galactose:*N*-acetylglucosamine galactosyltransferase activity was measured by incorporation of galactose into *N*-acetylglucosamine (44).

Immunological Methods

Cytochrome b_5 was solubilized from purified rat liver microsomes by trypsin digestion (10 μg trypsin/mg microsomal protein) at 4°C for 20 h and was purified by the method of Omura and Takesue (51). Antisera against cytochrome b_5 were produced in rabbits as described (18). For quantitative immunoprecipitation, purified rat liver ER and plasma membrane fractions were extracted with 1% Triton X-100 in 1 mM EDTA and 15 mM sodium 5,5-diethylbarbiturate buffer (pH 8.6) or were digested with trypsin as mentioned above. The extracts were adjusted to 2 mg protein/ml, mixed with 1 vol 0.5 M $NaHCO_3$ and 25 μCi [^{3}H]dimethylnaphthalene-5-sulfonyl chloride(dansylchloride) in 10 μl acetone, and incubated for 30 min at 37°C. Dansylation of the protein was stopped by addition of an excess amount of glutamine. ^{3}H-labeled membrane proteins were mixed with 10 μl anti-cytochrome b_5 serum in 0.5 ml phosphate buffered saline (PBS, pH 7.4) for 1 h at 37°C, mixed with 50 μl goat anti-rabbit-γ-globulin serum, and further incubated for 20 h at 4°C. Precipitates were washed three times with PBS and solubilized in 10 mM sodium phosphate buffer, pH 7.4, which contained 5% sodium dodecyl sulfate (SDS) and 10 mM mercaptoethanol. Aliquots were taken for liquid scintillation counting and for examination by SDS-polyacrylamide gel electrophoresis and autoradiofluorography (for details see reference 18).

Electron Microscopy

For electron microscope examinations, whole pellets as well as randomly chosen representative portions of the pelleted membrane fractions were prepared in buffer containing 0.3 M sucrose and 5 mM $MgCl_2$, fixed in 2.5% glutaraldehyde (50 mM sodium cacodylate buffer, pH 7.2, 2.5 mM $MgCl_2$, 50 mM KCl) for 30 min, washed with buffer, and postfixed with osmium tetroxide (2%, same buffer). Washing, dehydration, embedding, and ultrathin sectioning were done as described previously (20). Membranes were classified and relative membrane surfaces were determined by standard stereological methods (67).

Chemicals

NADH, NADPH, cytochrome *c*, trypsin, soybean trypsin inhibitor, and antimycin were obtained from Boehringer Biochemicals, Mannheim, Germany. Dextran and rotenone, which was postpurified by recrystallization from trichloroethylene, were from Sigma Chemical Co., St. Louis, Mo. Freund's complete adjuvant and goat anti-rabbit-γ-globulin serum were obtained from the Behringwerke, Marburg, Germany; UDP-1-[^{14}C]galactose and [^{3}H]dansylchloride from the Radiochemical Centre, Amersham, England. Other chemicals were analytical grade reagents from Merck, Darmstadt, Germany, or Serva, Heidelberg, Germany.

RESULTS

Morphology and Purity of Fractions

Throughout this study all fractions were examined by electron microscopy, and amounts of cross-contamination were routinely estimated. The rough microsomal fraction (Fig. 1 *a*) consisted of vesicles of a rather uniform size. More than 90% of the vesicles were associated with ribosomes which indicates that their origin is the rough ER (Table I). Treatment with buffers containing 1.5 M KCl, combined with sonication, removed most of the ribosomes and a considerable portion of intracisternal contents (Fig. 1 *b*; cf. Table II and reference 17). The smooth microsomal fraction consisted of ~90% smooth-surfaced vesicles (Table I).

Nuclear membrane fractions obtained in our laboratory have been extensively documented in previous articles (17, 20; for review see reference 16; cf. Table I).

Isolated Golgi apparatus usually showed three

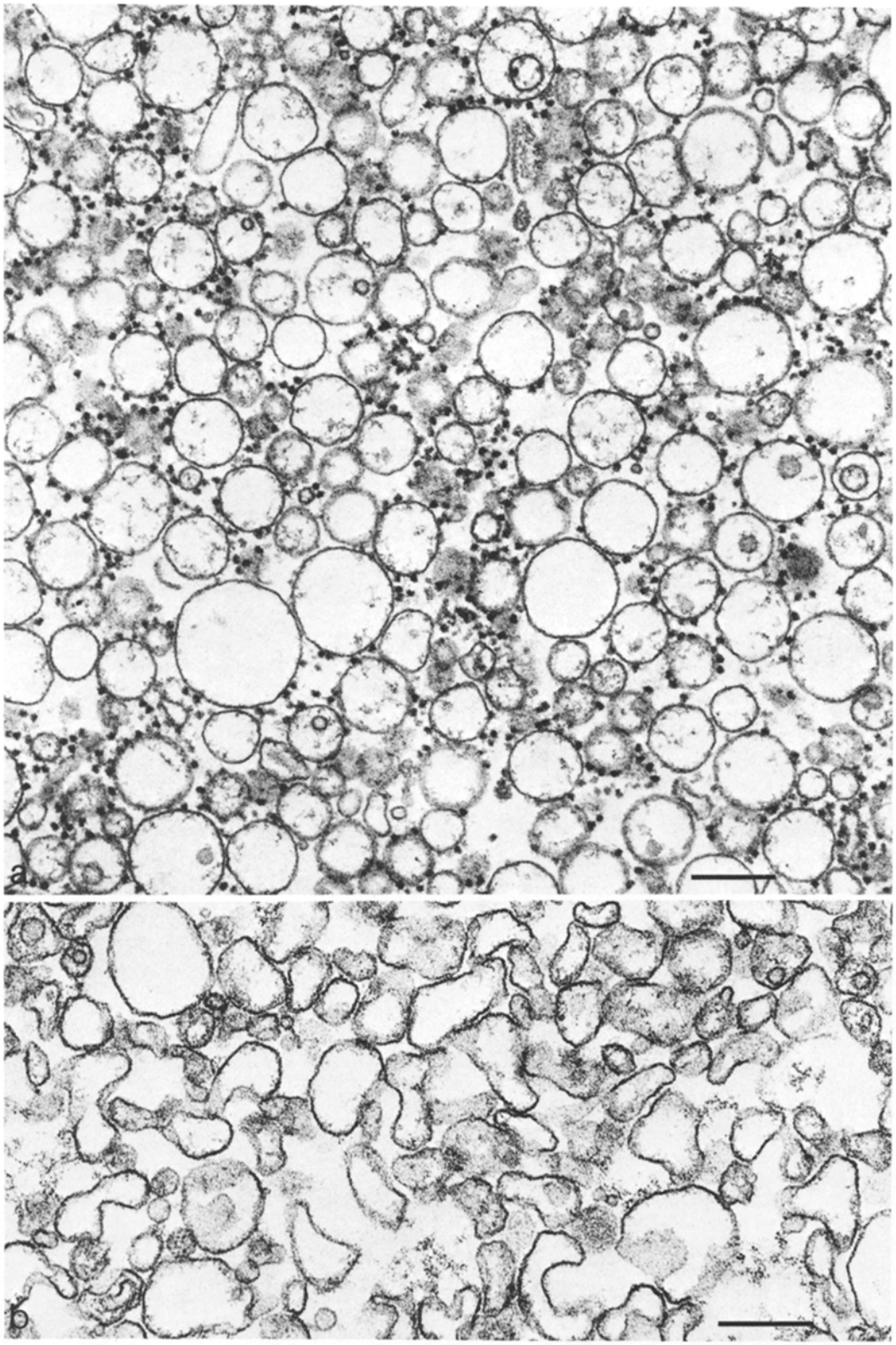

FIGURE 1 Survey electron micrograph of the fraction of rough microsomes (rough ER) from rat liver. Most of the vesicles show attached ribosomes (*a*) which are not present in fractions sonicated and treated with buffer containing 1.5 M KCl (*b*). Bars, 0.2 μm. (*a*) × 65,000; (*b*) × 75,000.

TABLE I

Morphometric Determinations of Membranous Contaminations in Ultrathin Sections through Rat Liver Fractions

Fraction	Per cent total membrane area						
	Nuclear	Rough ER	Mitochondrial	Plasma membrane	Dictyosomal		Other smooth membranes
Nuclei	90–95	3–4	0–0.5	0–1		2–4	
Rough ER	0–2	89–95	0–1	0–1		4–6	
Smooth ER	0–1	5–10	0–1	2–4		84–93	
Golgi apparatus	0–1	6–8	2–4	3–6	70–82		7–12
Plasma membranes	0–2	3–4	1–2	82–88		8–10	

For criteria of classification of the membranes see reference 20. The figures were determined from five different preparations of each fraction.

to five stacked cisternae filled with electron-dense material. Secretory sacs and vesicles which contained lipoprotein particles were still attached to dictyosomal stacks (cf. references 9, 13, and 47). Some peripheral cisternae and the secretory vesicles seemed to have swollen during the isolation procedure and appeared as enlarged empty vesicles (Fig. 2). Between 70 and 82% of the total membrane area was clearly identified as dictyosomal or secretory vesicle membranes. 7–12% of the profiles were smooth-surfaced membranes, possibly also of dictyosomal origin (Table I; cf. also Table II).

The plasma membrane fractions consisted mainly of pericanalicular membranes, including the canalicular microvilli and the associated junctional complexes (Fig. 3; for similar preparations see reference 20). Only small areas of sinusoidal surface were preserved as free membranous endings. The morphometric determinations showed that ~85% of all membrane profiles were clearly identified as plasma membrane, 8–10% were smooth-surfaced vesicles and sheets, most of which were probably also of plasma membrane origin (cf. Table II).

The purity of the fractions was also controlled by marker enzyme activities and chemical analyses (Table II). The phospholipid-to-protein ratio in ER and Golgi apparatus fractions increased by 20–30% after sonication and treatment with high salt concentrations, indicating the removal of some nonmembranous protein (see also Fig. 1 *a* and *b*). Rough and smooth ER were distinguished by their different RNA contents before high salt extraction. The high concentration of sialic acids in plasma membrane demonstrated the purity of this fraction (for references on the levels of sialic acids in endomembranes, see reference 20).

The specific activities of 5′-nucleotidase in the ER and Golgi apparatus fractions were ~5% of the activity in plasma membranes treated in parallel. The presence of this activity in the rough microsomal fraction may reflect some plasma membrane contamination (cf. the morphometry data) as well as the endogenous occurrence of this enzyme in rough ER (see reference 68).

The specific activities of glucose-6-phosphatase, a characteristic enzyme of hepatocyte ER and nuclear membrane, were only 3% in plasma membrane and 9% in Golgi apparatus fractions, compared to the activity present in the rough ER (cf. references 2, 13, and 47). Essentially the same specific activities were obtained when plasma membranes were isolated in the same media as used for ER preparation. When ER was isolated with essentially the same conditions as for plasma membrane isolation, the glucose-6-phosphatase activity was increased compared to the standard procedure (for the activation of glucose-6-phosphatase in alkaline solutions, see reference 61). These findings suggest that the low figures for glucose-6-phosphatase in plasma membranes are not caused by an inactivation produced during the different isolation procedures. The specific data for glucose-6-phosphatase activity in Golgi apparatus and plasma membrane fractions are in correspondence with the morphometrical data and indicate that most of the unidentified smooth membrane profiles (Table I) cannot be considered ER contamination.

UDP-galactose:*N*-acetylglucosamine galactosyltransferase is a preferential dictyosomal marker enzyme. In rough ER, 7% of the specific activity of Golgi apparatus was measured. The specific activities in the other fractions, especially in plasma membranes, were much lower. In all frac-

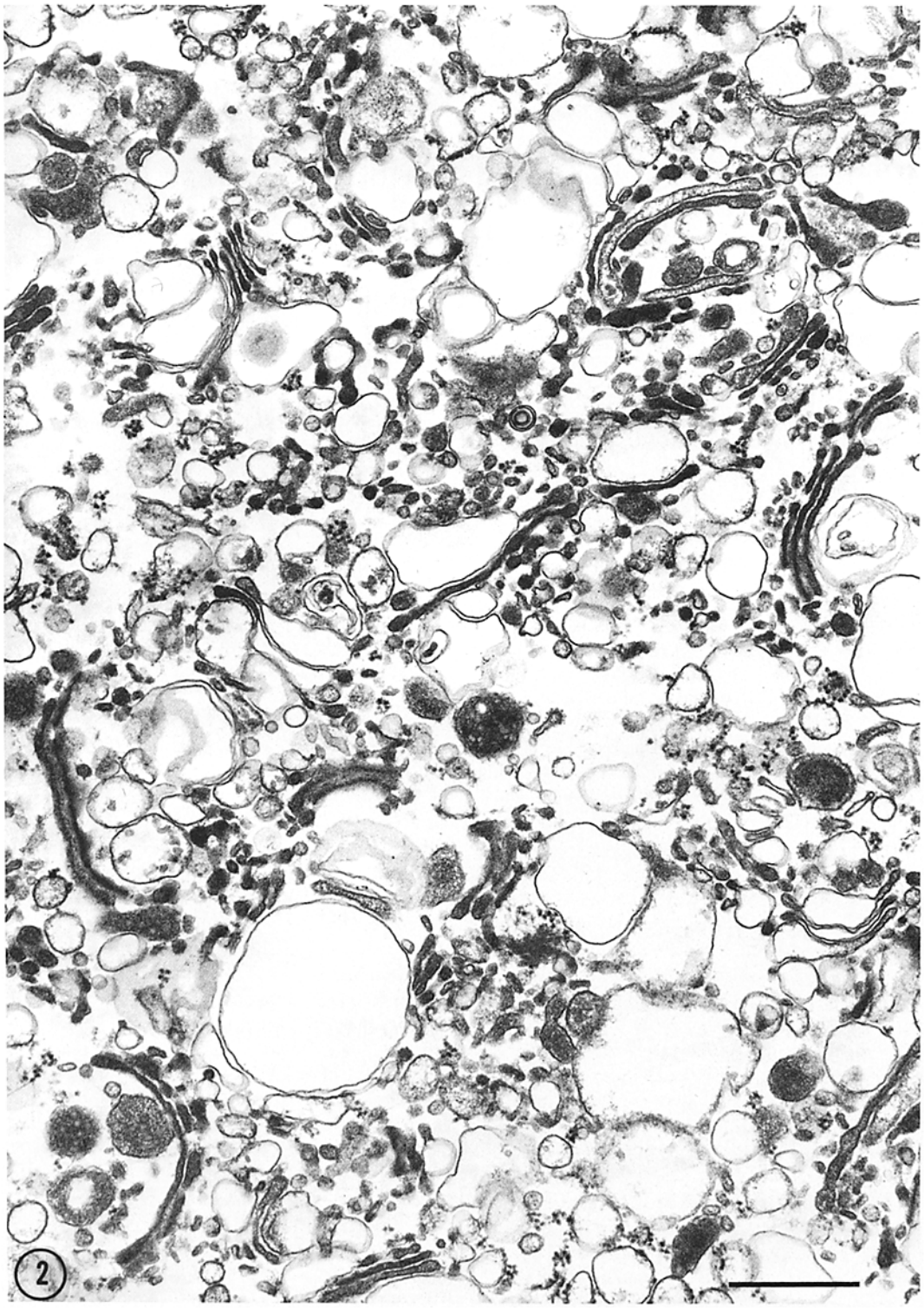

FIGURE 2 Survey micrograph of the fraction of Golgi apparatus. Stacked cisternae, their associated sacs, and secretory vesicles, most of which contain lipoprotein particles, are prominent. Bar, 0.5 μm. × 40,000.

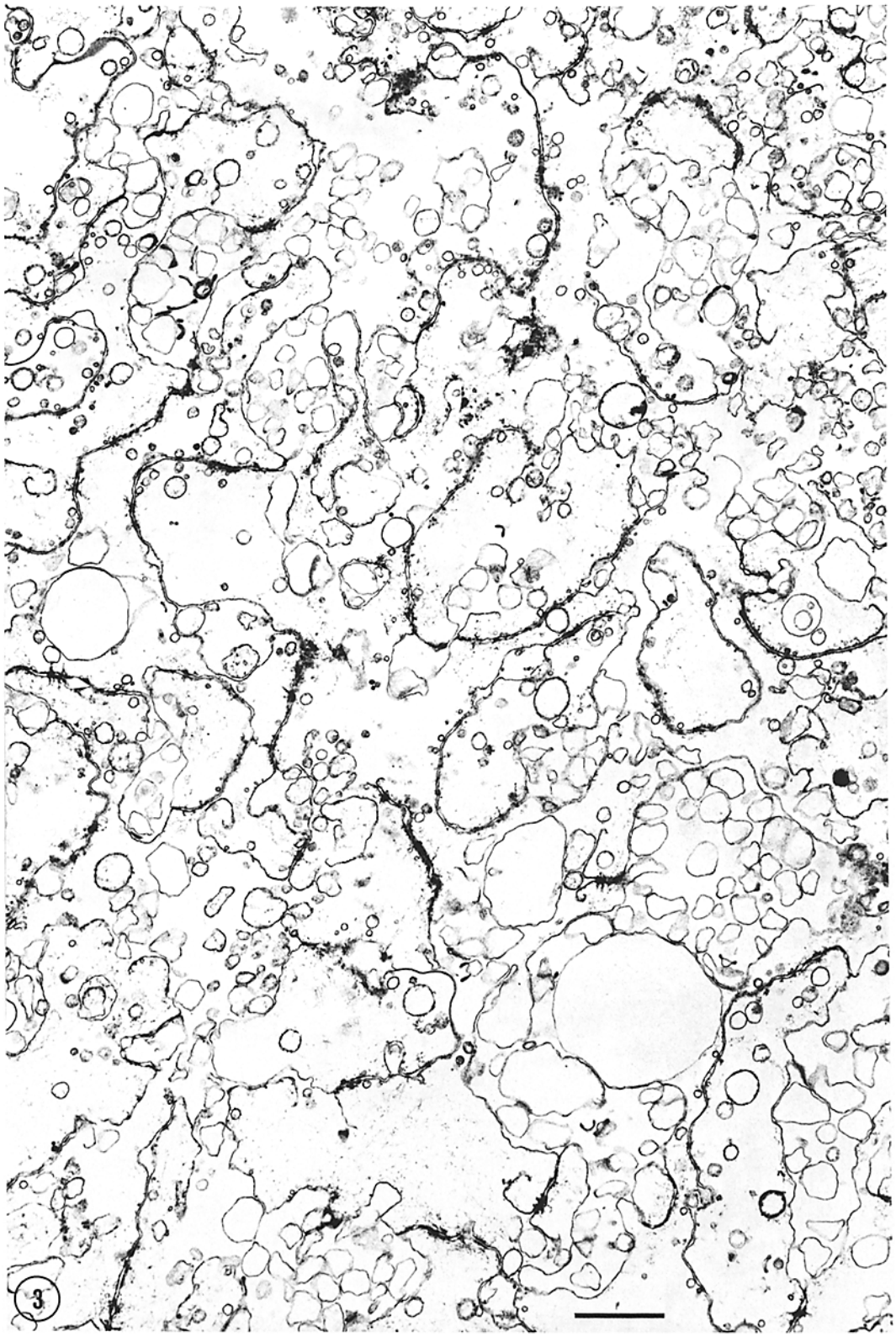

FIGURE 3 Survey micrograph of the fraction of plasma membranes from rat liver. The fraction is enriched in lateral cell membranes and includes junctional complexes, bile canaliculi, and canalicular microvilli. The microvillar membranes generally appear as saclike blebs. Note the retained association of such isolated membranes with some dense fibrillar material of the cell cortex, including desmosomal plates. Bar, 1.0 μm. × 14,000.

TABLE II

Characteristic Wt Ratios and Marker-Enzyme Activities of the Fractions

Fraction	Phospholipids / protein	RNA / phospholipid	Sialic acids / phospholipid	5'-Nucleotidase	Glucose-6-phosphatase	Galactosyl transferase	Cytochrome *c* oxidase
	wt/wt	*wt/wt*	*μg/mg*				
Nuclear membranes	0.32	0.15	1	0.07	0.35	0.2	0.02
Rough ER	0.37 (0.28)	0.11 (0.40)	6	0.14	0.89	1.1	0.04
Smooth ER	0.48 (0.32)	0.04 (0.09)	ND*	0.12	0.52	ND*	0.05
Golgi apparatus	0.50 (0.32)	0.02 (0.04)	12	0.14	0.08	15	0.12
Plasma membranes	0.45	0.015	39	3.0	0.03	0.1	0.10

Specific enzyme activities are expressed as: 5'-nucleotidase and glucose-6-phosphatase, μmol inorganic phosphate released/min × mg phospholipid; galactosyl transferase (UDP-galactose:*N*-acetylglucosamine galactosyltransferase), nmol galactose incorporated into exogenously added *N*-acetylglucosamine/min × mg phospholipid; cytochrome *c* oxidase, μmol ferrocytochrome *c* oxidized/min × mg phospholipid. Numbers in brackets correspond to fractions isolated in the presence of 5 mM $MgCl_2$ without extraction in high salt buffers. All other figures were determined from fractions after high salt treatment (see Isolation Procedures).

* Not determined.

tions studied, cytochrome *c* oxidase activity was very low, ranging from 0.3 to 2% of the activity of mitochondrial membranes (5.9 μmol cytochrome *c* oxidized/mg phospholipid × min) treated in parallel.

Spectral Analyses of Fractions

At the temperature of liquid nitrogen, difference spectra between dithionite-reduced versus oxidized plasma membranes showed absorbance maxima at 424, 448, 526, 552.5, 558, and 561 nm (Fig. 4). With NADH instead of dithionite used as reducing agent, only peaks at 423, 525, 552.5, and 558 nm were observed. These low temperature peaks are characteristic of the hepatic cytochrome b_5 (11, 23). In our ER fractions, both dithionite- (Fig. 4) and NADH-difference spectra only showed those peaks typical of cytochrome b_5. The spectra of the Golgi apparatus fractions were essentially identical to those of the plasma membranes. The maximum in the α-band region at 561 nm of the dithionite-difference spectra in plasma membrane and Golgi apparatus fractions could be attributed to cytochrome P-420 (for more detailed spectral analyses see reference 4). The presence of cytochrome P-420 in these fractions is further demonstrated by its absorbance maximum at 421 nm in the carbon monoxide difference spectra (Fig. 5). Cytochrome P-450 was also present in both membrane fractions as identified by the peak at 450 nm in the CO-

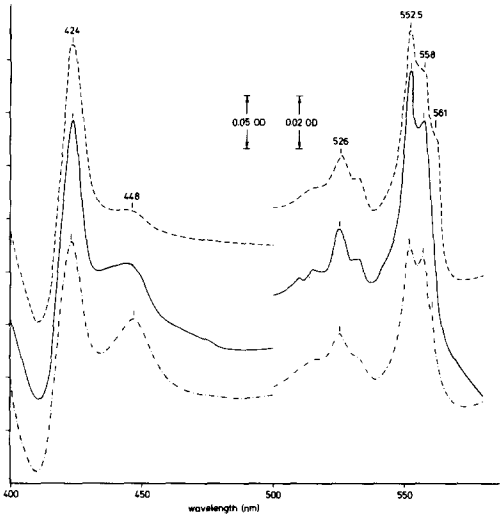

FIGURE 4 Dithionite-reduced versus oxidized difference spectra of isolated plasma membranes (- - -), Golgi apparatus (-·-·-), and rough ER (—) at −196°C. Optical path was 2 mm. Fractions (plasma membranes and Golgi apparatus, 1 mg membrane protein; rough ER, 0.25 mg membrane protein) were suspended in 1 ml of 0.5 M potassium phosphate buffer, pH 7.4, containing 50% glycerol. Other conditions were as described (4).

spectra. In the ER fractions, cytochrome P-420 was detected by a small shoulder at 421 nm of the high peak of cytochrome P-450. The maximum at ~448 nm (Fig. 4) was not caused by the presence

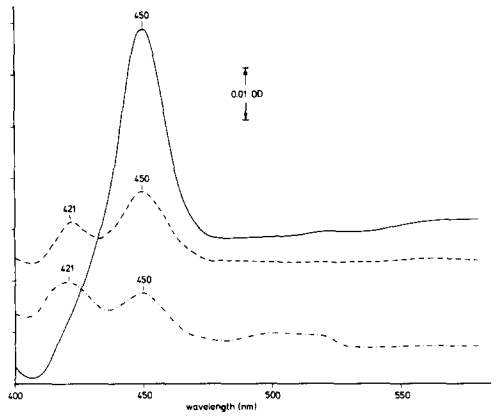

FIGURE 5 Carbon monoxide difference spectra of isolated plasma membranes (- - -), Golgi apparatus (-·-·-), and rough ER (—) at room temperature. Protein concentrations were 1 mg/ml. Other conditions were as described (4).

of cytochrome aa_3, as was evident from the complete absence of: (*a*) a band at 605 nm, (*b*) cytochrome oxidase activity (Table II), and (*c*) other mitochondrial redox components (cytochromes *c* and c_1, succinate dehydrogenase). The 448-nm peak was not apparent in difference spectra obtained with ascorbate as electron donor (as is cytochrome aa_3), and after the addition of dithionite it developed gradually during ~30 min-incubation at room temperature. It was also observed in frozen and thawed ER membranes (see the low temperature difference spectra, Fig. 4) and in ER membranes that had been stored for prolonged periods. This peak possibly reflects the binding of endogenously formed CO to cytochrome P-450 (cf. reference 48). In both CO- and dithionite-spectra, hemoglobin was not detected in any of our purified fractions.

At 20°C the spectral peaks of the NADH-difference spectra were at identical wavelengths (425, 528, and 556 nm) in all fractions studied. After addition of dithionite or ascorbate, the Soret- and α-bands shifted by 2 nm to longer wavelengths (to 427 and 558 nm, respectively) in Golgi apparatus and plasma membranes, but not in the ER membranes. This shift is explained by the spectral overlap of cytochrome b_5 with cytochrome P-420, which is reduced by dithionite and ascorbate, but not by NADH (50), and has absorbance maxima at 428, 530, and 560 nm.

The concentrations of the three cytochromes in rat liver fractions, calculated on a phospholipid basis, are summarized in Table III. The highest contents of the cytochromes b_5 and P-450 were observed in rough and smooth ER fractions. Since cytochrome P-420 is usually regarded as an inactive modification of cytochrome P-450, we also compared the sum of these cytochromes in the fractions.

Cytochromes b_5 and P-450, but not cytochrome P-420, have been previously demonstrated in rat liver nuclear membranes (17, 33, 36). However, when we examined isolated nuclei we found relatively high cytochrome P-420 concentrations. The low values obtained in the nuclear membrane fractions were probably caused by degradation during the preparations. The cytochrome b_5 concentration in nuclear membranes was almost as high as in isolated nuclei. In both Golgi apparatus and plasma membranes the contents of cytochrome b_5 and of cytochromes P-450 + P-420 were 27–36% of those in rough ER. These figures are too high to be explained by contamination with ER elements (see Tables I and II). The cytochrome P-420 contents were similar in all fractions (between 240 and 480 pmol/mg phospholipid), except for the nuclear membranes.

NADH- and NADPH-Cytochrome c Reductase Activities

Activities of rotenone- and antimycin-insensitive NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase were detected in all fractions (Table III). The rough and smooth ER fractions contained by far the highest activities of both enzymes. In the Golgi apparatus membranes, the specific activity of the NADH-dependent enzyme was 29% of that in the rough ER, and the NADPH-dependent specific activity was only 13%. In plasma membranes both enzyme activities ranged from 10 to 12% of that in the rough ER. These low figures might be partly explained by inactivation, as suggested from the rapid decrease of rotenone-insensitive NADH-cytochrome *c* reductase activity in isolated plasma membranes during prolonged storage at 4°C (cf. references 2, 8, 10, 31, and 43). In both nuclei and nuclear membranes the NADH-dependent enzyme activities were ~25% of that of the rough ER.

Extraction of Cytochromes

Cytochrome b_5 can be extracted from microsomal membranes by trypsin digestion (34, 51; see also Materials and Methods) or by treatment

TABLE III

Redox Components in Endomembranes and Plasma Membranes of Rat Liver

Fraction	Cytochrome contents			Rotenone-insensitive NADH-cytochrome *c* reductase	NADPH-cytochrome *c* reductase
	b_5	P-450	P-420		
Nuclei (6)	0.85	0.60	0.48	0.75	0.13
Nuclear membranes (25)	0.54	0.48	0.03	0.82	0.04
Rough ER (30)	1.55	1.75	0.27	3.19	0.30
Smooth ER (12)	1.37	1.30	0.33	2.80	0.32
Golgi apparatus (15)	0.56	0.40	0.24	0.93	0.04
Plasma membranes (26)	0.45	0.28	0.26	0.38	0.03

Cytochrome concentrations are expressed as nmol per mg phospholipid. Enzyme activities are expressed as μmol ferricytochrome *c* reduced/min per mg phospholipid. Phospholipid contents of 40–60 μg/mg protein were determined for isolated nuclei. For the phospholipid contents of the other fractions see Table II. All membrane fractions had been extracted with high salt concentrations. Number of experiments from which the data are compiled are given in brackets.

with low detergent concentrations (60). Under these conditions, cytochromes P-450 and P-420 remain firmly bound to the membrane (cf. references 42 and 51). These procedures also allow a separation of cytochrome b_5 from cytochromes P-450 and P-420 in plasma membranes. Cytochrome b_5 solubilized from plasma membranes and the insoluble cytochrome P-420 were spectrophotometrically indistinguishable from the corresponding ER pigments. A quantitative estimation of the specific contents of the cytochrome b_5 and P-420 from the recoveries of these treatments was not possible because of degradation (34% in the case of cytochrome b_5 and 70% in the case of cytochromes P-450 + P-420). Under the same preparative conditions, the cytochromes of ER membranes were more stable (cytochrome b_5, 5% degradation; cytochromes P-450 + P-420, 47% degradation). Extraction of plasma membranes with 1% Triton X-100 yielded a better cytochrome b_5 recovery, but resulted in a complete loss of spectrophotometrically detectable cytochrome P-450 in the residual membrane material.

Stability of Cytochromes

We have examined the possibility that the relatively high amounts of cytochrome P-420 observed in isolated plasma membranes, Golgi apparatus, and nuclei were because of conversion of cytochrome P-450 during the preparations. (*a*) Different buffers and salts in the isolation media did not convert microsomal cytochrome P-450 into P-420 (see Materials and Methods). Repeated washings of ER membranes in large volumes of the buffer used for the plasma membrane isolation did not affect the cytochrome P-450 and P-420 contents. (*b*) When ER and plasma membranes were extracted with high salt buffers, cytochrome b_5 was almost completely recovered, whereas some conversion of cytochrome P-450 to P-420 occurred (Table IV). In plasma membranes there was also evidence for degradation of both cytochromes. Under such conditions, dramatic degradation of both cytochromes was found in nuclei and nuclear membranes (Table IV), a marked though yet unexplained phenomenon (cf. also the data in reference 1). (*c*) Prolonged incubation in sucrose solutions resulted in decreased contents of cytochrome b_5 in all fractions (Table IV). The cytochrome P-450 content of rough ER was also diminished, and a considerable proportion seemed to be converted into cytochrome P-420. In plasma membranes, however, cytochrome P-450 was completely lost, and cytochrome P-420 was decreased by ~30%. When the incubation temperature was increased from 4° to 10°C, the degradation of the cytochromes was higher in all fractions. (*d*) When the fractions had been frozen and thawed (Table IV), the cytochrome b_5 contents were essentially unaffected. A slight decrease was observed in the ER of cytochromes P-450 and P-420 in contrast to drastically reduced contents in plasma membranes and nuclei. (*e*) Addition of catalase and of benzoic acid to the isolation media, to avoid peroxidation of membrane lipids (26), had no effect on the cytochrome contents. A possible oxidation of sulfhydryl groups of the membrane proteins was prevented by the presence of 2 mM mercaptoethanol in the solutions. Endogenous protease activity was inhibited by the presence of 0.1 mM phenyl meth-

TABLE IV

Stability of the Spectrophotometrically Detectable Cytochromes in Rat Liver Fractions under Various Experimental Conditions

Fraction and treatment	Cytochrome concentrations			
	b_5	P-450	P-420	P-450 + P-420
	(nmol/mg phospholipid)			
Rough ER				
Control	1.57 (100)	2.14 (100)	0.15 (100)	2.29 (100)
Extraction in 1.5 M KCl for 1 h, wash	1.54 (98)	1.81 (85)	0.27 (184)	2.07 (90)
Incubation in 0.25 M sucrose for 24 h	1.00 (64)	1.22 (57)	0.36 (240)	1.58 (69)
Freezing at −20°C for 24 h, and thawing	1.48 (94)	1.88 (88)	0.12 (83)	2.00 (87)
Incubation with 1% Triton X-100 for 1 h	1.32 (84)	0.15 (7)	0.10 (65)	0.25 (11)
Incubation with 0.13% deoxycholate for 1 h	1.38 (88)	0.58 (27)	0.44 (293)	1.02 (45)
Plasma membrane				
Control	0.47 (100)	0.39 (100)	0.30 (100)	0.69 (100)
Extraction in 1.5 M KCl for 1 h, wash	0.45 (95)	0.28 (72)	0.26 (87)	0.54 (78)
Incubation in 0.25 M sucrose for 24 h	0.35 (75)	0 (0)	0.21 (71)	0.22 (32)
Freezing at −20°C for 24 h, and thawing	0.41 (87)	0.09 (24)	0.11 (36)	0.20 (29)
Incubation with 1% Triton X-100 for 1 h	0.26 (55)	0 (0)	0.29 (97)	0.29 (42)
Incubation with 0.13% deoxycholate for 1 h	0.45 (96)	0.13 (33)	0.11 (38)	0.24 (35)
Nuclei/Nuclear membrane				
Control nuclei	0.83 (100)	0.60 (100)	0.48 (100)	1.08 (100)
Nuclei extracted in 1.5 M KCl for 1 h, wash (crude nuclear membranes)	0.63 (48)	0.47 (79)	0 (0)	0.47 (26)
Incubation in 0.25 M sucrose for 24 h	0.62 (75)	0.45 (75)	0.18 (38)	0.63 (58)
Freezing at −20°C for 24 h, and thawing	0.75 (90)	0.47 (79)	0.04 (9)	0.51 (47)
Incubation with 1% Triton X-100 for 1 h	0.42 (50)	0.32 (54)	0.06 (12)	0.38 (35)

Values in brackets give recoveries of the cytochrome components after the specific treatments, expressed as percent of the amounts present in the control. All treatments were performed in 10 mM Tris-HCl buffer, pH 7.4, at 4°C; protein concentrations of the fractions were 3–5 mg/ml. Recoveries of phospholipids after high salt extraction were almost 100% in the case of rough ER and plasma membranes, but only 45–50% in the case of nuclei.

ane sulfonylfluoride. Both reagents had no measurable effects on the cytochrome contents. (*f*) To inhibit possible phospholipase A activity (e.g. reference 66), Ca^{2+} was omitted in the isolation media and NaF was added at 1-mM concentration without, however, any stabilizing effect on plasma membrane cytochromes. (*g*) After incubation of the fractions with Triton X-100 cytochrome P-450 contents were greatly reduced; in contrast cytochromes b_5 and P-420 were much more stable, except in the nuclei. Treatment with low concentrations of sodium deoxycholate (cf. references 20 and 38) resulted in moderately decreased cytochrome P-450 contents in both rough ER and plasma membranes (Table IV), obviously because of the rapid conversion into cytochrome P-420 (cf. reference 50). In dithionite-difference spectra of deoxycholate-treated ER membranes, the absorbance maxima showed the same characteristic shift towards longer wavelengths as untreated plasma membranes (Fig. 4) because of the increased cytochrome P-420 contents. In nuclei and plasma membranes treated with deoxycholate, contents of cytochrome P-420 were very low, indicative of the higher degradation of this pigment in nuclear and plasma membranes already described above. (*h*) When purified plasma membranes were mixed with various defined amounts of rough ER membranes and incubated for 24 h, the amounts of the specific cytochromes present in these mixtures were always the same as in the individual fractions held separately under identical conditions. This shows that the isolated plasma membrane material does not affect the cytochrome contents of rough ER and suggests that the "destabilizing factor" for cytochrome P-450

present in plasma membranes acts only on the plasma membrane-bound cytochromes.

Immunological Studies

Immunoglobulins obtained from rabbit antisera against cytochrome b_5 from rat liver microsomes have been shown to inhibit the NADH-cytochrome *c* reductase activity of microsomal (53) and Golgi apparatus (3) fractions from rat liver. We found a similar inhibition of the NADH-cytochrome *c* reductase activity in isolated plasma membranes and in rough ER (Fig. 6). When purified trypsin-resistant cytochrome b_5 fragments were added to ER membranes, this inhibition was partially overcome, which indicates that the antibody was acting at the cytochrome b_5 site of the enzyme complex. In both plasma membranes and rough ER, the NADPH-cytochrome *c* reductase activity was not inhibited by the addition of antibodies to cytochrome b_5. In Ouchterlony double immunodiffusion tests (Fig. 7), precipitation was observed between antibodies to cytochrome b_5 and plasma membrane material released by tryp-

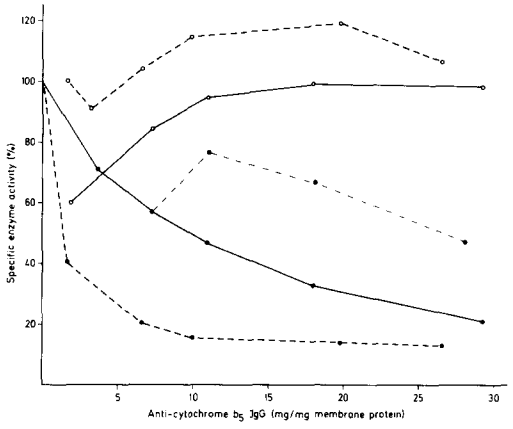

FIGURE 6 Effect of rabbit anti-cytochrome b_5 immunoglobulin on NADH-cytochrome *c* reductase (—●—) and NADPH-cytochrome *c* reductase (—○—) activities in rough ER membranes (solid lines) and plasma membranes (dashed lines) from rat liver. Membranes were incubated for 1 h at 25°C with purified immunoglobulins from a rabbit antiserum raised against the trypsin-resistant fragment of cytochrome b_5 from rat liver ER (anti-cytochrome b_5 IgG) or with the same amount of a control rabbit immunoglobulin fraction. Aliquots of the suspension were then used for the enzyme assays. Enzyme activity in the presence of anti-cytochrome b_5 IgG is expressed as a percentage of that activity in the presence of the corresponding amount of the control IgG. When 5 μg purified cytochrome b_5 was added to rough ER membranes which had been incubated with anti-cytochrome b_5 IgG, enzyme activity was partially restored (-·-·-).

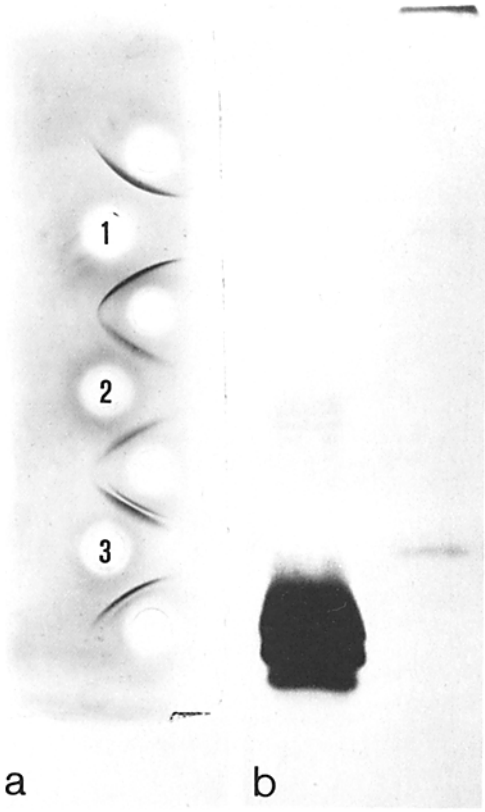

FIGURE 7 (*a*) Ouchterlony double diffusion test in which purified immunoglobulins from a rabbit antiserum raised against the purified trypsin-released cytochrome b_5 fragment from rat liver rough ER (anti-cytochrome b_5 immunoglobulin G) have been allowed to react with total trypsin-released material from rat liver plasma membranes and from rough ER. The four wells in the right each contained 0.2 mg anti-cytochrome b_5 IgG in 20 μl 5,5-diethylbarbiturate buffer (75 mM, pH 8.6). The wells in the left contained (*1*) 0.02 mg purified cytochrome b_5 fragment, (*2*) 0.165 mg of plasma membrane material, and (*3*) 0.15 mg of rough ER material. Note precipitin reaction in material released from plasma membrane. (*b*) Precipitates from Triton X-100-solubilized material of isolated rat liver plasma membranes with anti-cytochrome b_5 IgG (right slot) as revealed in autoradiofluorographs after ^{3}H dansylation of the membrane material and separation by SDS-polyacrylamide gel electrophoresis, in comparison with the precipitate formed with the purified trypsin-released cytochrome b_5 fragment (left slot). Note the greater mobility of the cytochrome b_5 fragment.

sin digestion. Continuity of precipitin lines was noted between cytochrome b_5 of ER and of plasma membrane, which indicates immunological identity of the pigments present in both membranes. When we determined the amount of cytochrome b_5 in Triton X-100-solubilized plasma membrane material by precipitation with antibodies to microsomal cytochrome b_5, we obtained an immunoprecipitate representing 30–35% of the relative amount of cytochrome b_5 precipitated from solubilized ER material. This figure corresponds to the spectrophotometrically determined cytochrome b_5 contents (Table III). On gel electrophoresis, cytochrome b_5 present in Triton X-100 extracts of plasma membranes showed the same mobility as one of the cytochrome b_5 components of rough ER membranes. On gel electrophoresis of cytochrome b_5 from rough ER membranes, we frequently observed two major different cytochrome b_5 components (Fig. 8*a* and *b*; cf. reference 18), in agreement with previous results of various other authors (15, 34, 53, 54, 56). This heterogeneity of microsomal cytochrome b_5 was found with both trypsin-released and Triton-solubilized material. In contrast, we detected only one component in the Triton-solubilized plasma membrane material; this component precipitated with antibodies to total microsomal cytochrome b_5 which had the same electrophoretic mobility as the slower cytochrome b_5 component from ER (Fig. 8*b*). This also supports our conclusion that the cytochrome b_5 observed in plasma membrane fractions does not represent contamination by ER elements.

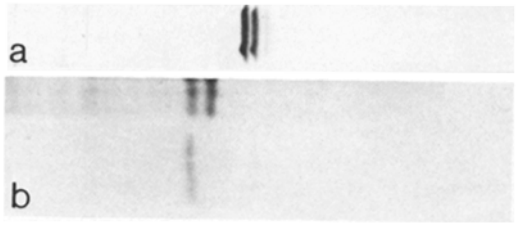

FIGURE 8 SDS-polyacrylamide gel electrophoresis of immunoprecipitates of trypsin-released material from isolated rat liver rough ER with antibodies to rat liver microsomal cytochrome b_5. (*a*) Bands stained with Coomassie blue. Note the two distinct major and one faint minor components (cf. references 15, 20, 54, and 56). (*b*) Autoradiofluorographs of immunoprecipitates from total Triton X-100-solubilized and ^{3}H-dansylated material of isolated rat liver rough ER (upper slot) and plasma membranes (lower slot) after specific precipitation with anticytochrome b_5 IgG. Note that only one cytochrome b_5 component is identified in the plasma membrane material, in contrast to the two major components present in rough ER.

DISCUSSION

The ER forms a dynamic cisternal system which functionally, and often morphologically, appears to be interconnected with the nuclear envelope, membranes of the Golgi apparatus, outer membranes of mitochondria and plastids, and various vesicles such as peroxisomes and lysosomes (for reviews see references 7, 16, 19, and 47). These interrelationships may explain the occurrence of a diversity of proteins and enzymes common to all these membranes, although they are often present in lower concentrations than in the ER. The occurrence of such components in plasma membranes, however, is subject to debate. An especially well-characterized example of ER membrane proteins are the components of the microsomal electron transport systems in mammalian liver. In plasma membrane fractions from rat liver, most authors have negatively reported the presence of significant amounts of cytochrome b_5 and cytochromes P-450 and P-420 (10, 14, 15, 28). Vassiletz et al. (65) have postulated the presence of cytochrome b_5 together with cytochrome *c*, but not cytochrome P-450, in rat liver plasma membranes; however, the concentrations given by these authors (9% of the cytochrome b_5 contents of their microsomal fraction) are identical to the ER contamination reported.

It is obvious that the question as to whether relatively small amounts of these cytochromes are truly endogenous to plasma membranes is difficult to answer in view of the inevitable contamination of plasma membrane fractions with endomembrane material. From our present study, however, we conclude that for the following reasons the cytochromes b_5, P-450, and P-420 are endogenous to plasma and Golgi membranes:

(*a*) We have consistently found, in highly purified plasma membrane fractions, a nearly constant cytochrome b_5 content of ~30% of that present in rough ER fractions isolated in parallel. This figure is far too high to be explained by contamination with ER elements, as judged from morphometry and determination of marker enzyme activities (see Tables I and II).When the cytochrome b_5 concentrations determined in our fractions are corrected for maximal cross-contamination by other membrane elements, a minimum cytochrome b_5 content endogenous to both Golgi

apparatus and a plasma membrane of ~20% of that present in rough ER is estimated. When a similar estimation is made for the contents of cytochromes P-450 and P-420, again a relative content in plasma membrane and Golgi apparatus of ~20% of the level found in rough ER is obtained.

(*b*) Our studies suggest immunological identity of the cytochrome b_5 found in ER with that found in plasma membrane fractions, as judged from double diffusion tests of membrane extracts and from gel electrophoresis of the solubilized membrane material immunoprecipitated with antibodies against ER-cytochrome b_5. However, our immunological findings also strongly suggest that plasma membrane cytochrome b_5 differs from ER cytochrome b_5 by the absence of a second molecular category characterized by higher electrophoretic mobility on SDS-polyacrylamide gels (see Fig. 8 and references quoted in Results). This again speaks for the significance of plasma membrane-associated cytochrome b_5 and provides another argument against its originating from contaminating ER elements.

(*c*) The endogenous character of the plasma membrane-bound cytochromes P-450 and P-420 is also indicated by the different ratio of both pigments, compared to ER, and by the differential instability of cytochrome P-450 in the plasma membrane (see Table IV). Under a variety of experimental conditions, plasma membrane cytochrome P-450 is much more rapidly degraded than the cytochrome P-450 present in ER membranes. In addition, cytochrome P-420, which is present in similar concentrations in all membranes studied (except for the high-salt-treated nuclear membranes), shows a much higher susceptibility to degradation in plasma membrane fractions as compared to rough microsomes, with the possible exception of the treatment with Triton X-100 (Table IV).

(*d*) The presence of cytochromes b_5 and P-420 in liver plasma membranes is not without precedence in other cell systems. We have observed significant amounts of these cytochromes in milk fat globule membranes (4, 31), human erythrocyte ghosts[1] (see also reference 27), and microvillar membranes of chicken intestinal brush border.[2] In addition, Charalampous et al. (5) have reported in plasma membrane fractions isolated from cultured human carcinoma cells (KB) relatively high levels of cytochrome b_5, as compared to the levels in their microsomal fractions. These authors did not find appreciable amounts of cytochrome P-450 in both their plasma membrane and microsomal fractions but reported surprisingly high levels of NADPH-cytochrome *c* reductase activities in their plasma membrane fractions. In discussing our observations of cytochromes b_5 and P-450 + P-420 in plasma membranes and Golgi apparatus from rat liver, it is also worth mentioning that b-type cytochromes are rich in a well-studied secretory vesicle, the chromaffin granule from the adrenal medulla (see reference 58).

Our conclusions are based on the assumption that possible ER contamination present in Golgi apparatus and plasma membrane fractions are similar to ER elements recovered in microsomal fractions. The data of our experiments on the stability characteristics of various components such as cytochromes b_5, P-450, and P-420, NADH- and NADPH-cytochrome *c* reductases (cf. references 2, 10, 26, 31, and 43), and especially the observed stability of glucose-6-phosphatase activity (cf. references 35 and 61) under exactly parallel conditions as used for plasma membrane isolation, support this assumption. We cannot exclude, however, the radical alternative that our plasma membrane and Golgi apparatus fractions might have been contaminated by a special ER subfraction with properties different from that of our ER fractions, e.g., which results from selective partial degradation.

Our conclusions of the presence of cytochromes b_5 and P-450 + P-420 in Golgi apparatus and plasma membranes seem to be in conflict with data of other authors (see Introduction). However, negative results reported in the literature might well have been influenced by the selective degradation described in this study and (or) the enrichment of selected plasma membrane domains. Fowler et al. (15) have reported that a ferritin-coupled anti-cytochrome b_5/anti-ferritin hybrid antibody (55) did not decorate their isolated Golgi apparatus and plasma membranes, and they have interpreted this as demonstrating absence of cytochrome b_5 in these membranes. In discussions of the observations of these authors, one must keep in mind that their procedure allowed the detection of only 5% of total cytochrome b_5 present. Negative results obtained with

[1] Bruder, G., and E.-D. Jarasch. Manuscript in preparation.

[2] Bruder, G., E.-D. Jarasch, and A. Bretscher. Unpublished observations.

immunolocalization techniques in general cannot be regarded as conclusive demonstration of the absence of a specific component because of problems of differences in accessibility (see also reference 45) and specific arrangement of the antigenic determinant. In addition, the specific isolation methods used by these authors (15, 55), which involve the use of detergents (Triton WR-1339, digitonin) in some of their preparations, might have contributed to removal or masking of antigenic determinants.[3] Preliminary electron microscope studies in our laboratory with monospecific antibodies to microsomal cytochrome b_5 and immunoperoxidase labeling techniques on frozen sections of rat liver tissue, have shown positive decoration of pericanalicular plasma membranes almost as intensely stained as in the ER cisternal membranes.[4] The different regions of the hepatocyte plasma membrane differ not only morphologically but also in their biochemical composition (e.g. reference 39). The method for isolating liver plasma membranes used by ourselves and other authors (10, 14, 15, 28, 39) are highly selective for pericanalicular plasma membranes and include junctional complexes and bile canalicular microvilli. Thus, we cannot exclude that other regions of the hepatocyte plasma membrane contain lesser or greater amounts of cytochromes.

Nothing can be said at the moment of the special biological functions of these redox components in the Golgi apparatus and the plasma membrane. Quantitatively, the contribution of the plasma membrane cytochromes to the total hepatocyte cytochromes obviously is minuscle and thus not critical for general redox reactions that could as well be carried out in the ER system. Special roles for the cytochromes in the membranes, however, are conceivable. For example, an involvement of such redox components in the hormone stimulation of plasma membrane-bound enzymes has been postulated (6). Finally, one must also consider the possibility that the presence of these pigments in the Golgi apparatus and the plasma membrane reflects the inclusion of these components in exocytotic processes followed by their appearance, perhaps only transitorily, in the surface membrane (for discussion see also references 2, 19, 21, 38, and 47).

[3] When our plasma membranes were incubated with low concentrations of digitonin, >50% of the cytochrome b_5 was extracted into the supernate, and cytochromes P-450 and P-420 were no longer detected.

[4] Krohne, G., G. Bruder, A. Fink, E.-D. Jarasch, and W. W. Franke. Manuscript in preparation.

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