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ISOLATION AND CHARACTERIZATION OF ROUGH ENDOPLASMIC RETICULUM ASSOCIATED WITH MITOCHONDRIA FROM NORMAL RAT LIVER

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A subfraction of rough endoplasmic reticulum (RER) characterized by its close association with mitochondria (MITO) was isolated from low speed pellets of normal rat liver homogenate under defined ionic conditions. This fraction enriched in MITO-RER complexes contained 20% of cellular RNA, 20% of glucose-6-phosphatase and 47% of cytochrome *c* oxidase activities. Morphologically, the isolated MITO-RER complexes closely resembled physiological associations between the two organelles commonly seen in intact liver. Partial dissociation of RER from mitochondria of the MITO-RER fraction was achieved by either EDTA (0.5 mM) or by hypotonic/hypertonic treatment of MITO-RER complexes. With the latter procedure approx. 70% of RER (RER_{mito}) with 50% of ribosomes still attached could be separated from the inner compartments of mitochondria. This RER_{mito} exhibited a higher glucose-6-phosphatase activity than RER isolated as rough microsomes from the postmitochondrial supernatant. Isopycnic centrifugation on linear metrizamide gradients revealed that the mitochondria-associated part of RER corresponds to the high density, ribosome-rich subfraction of rough microsomes isolated in cation-free sucrose solution. The combined data demonstrate that a morphologically and biochemically distinct portion of RER is associated with mitochondria and support the concept of considerable intracellular heterogeneities in distribution of enzymes and enzyme systems along the lateral plane of the endoplasmic reticulum membrane system.

Introduction

Ultrastructural studies of mammalian liver have repeatedly emphasized that with respect to its intracellular topological arrangement mainly two portions of rough endoplasmic reticulum (RER) can be distinguished within hepatocytes, one portion being arranged in parallel arrays of broad flattened bags (cisternae) whereas the other portion of intracellular RER consists of single cisternae intimately associated with mitochondria [1–5]. It has been suggested that the ratio between the two types of RER depends on the physiological state of cells [6–8] and varies between subpopulations of hepatocytes [4,9]. In addition, for adult rat liver a special regulatory role of the mitochondria associated part of RER (RER_{mito}) during the rapid proliferation of hepatic endoplasmic reticulum has been proposed [10]. However, since all these studies were morphological in nature the important question whether the obviously heterogeneous intracellular distribution of RER is simply a random phenomenon or indeed mirrors a specific functional compartimentalisation of this organelle is unknown.

Numerous subfractionation studies performed in recent years suggest that in quantitative terms many enzymes and enzyme system are heterogeneously distributed along the lateral plane of the RER membrane system [11–15]. However, since homogenisation of rat liver tissue disintegrates RER into membrane fragments of variable sizes and densities which sediment anywhere between 100 and 100 000 $\times g$, the

question whether rough microsomes, i.e. RER contained in a postmitochondrial supernatant, as commonly used in biochemical studies are representative for the whole spectrum of hepatic RER occurring in vivo, is controversial [15,17]. Furthermore, since the morphology of rough microsomes is vastly different from the structure of RER in vivo and since the numerous variations of subcellular fractionation conditions used by different investigators obviously all lead to variable degrees of sorting out of the heterogenous RER membranes [18–22] the degree and type of reported heterogeneities also vary considerably [15–17] and the different subfractions of rough microsomes cannot be identified with respect to their intracellular origin. This however should be possible if at least one part of RER could be preferentially isolated in intact unaltered form.

Several investigations from this and other laboratories have provided evidence that mitochondria-associated RER (RER_{mito}) can indeed be isolated and preserves its bilammellar configuration and its close structural association with mitochondria (MITO-RER complexes [22–25]). The careful centrifugation data of Shore and Tata [22] and more recently of Pickett et al. [25] have provided good evidence for a structural association between the two hepatic organelles. However, the physiological importance of this association remains poorly understood. With the ultimate purpose to elucidate an apparent relationship between the structural contact and the function of mitochondria and RER, we therefore have adapted the differential centrifugation procedure previously used in rats treated with cobaltous chloride ($CoCl_2$ [24]) for the selective isolation of RER_{mito} from normal rat liver. In addition, in order to correlate already reported in vitro heterogeneities between various subfractions of rough microsomes [12,15] to intracellular heterogeneity of RER in vivo, RER_{mito} was recovered from low speed pellets of liver homogenates prepared under defined ionic conditions and was enzymatically characterized and compared to subfractions of rough microsomes prepared exclusively with cation-free sucrose solutions according to well established fractionation procedures [26].

The data presented in this study demonstrate that a distinct subtype of RER membranes is associated with MITO-RER complexes. Biochemically, RER_{mito} is characterized by a high specific activity of glucose-6-phosphatase and appears to correspond to the high density, ribosome rich subfraction of RM described by other investigators [12,15,27]. These findings further support the concept of intracellular functional heterogeneity of RER and are consistent with the view that in vitro heterogeneities of endoplasmic reticulum membranes either with respect to their sedimentation behaviour or to their enzymatic composition may reflect differences in the structure and function of endoplasmic reticulum in vivo [17] rather than differences in the physical property of these membranes in vitro [13,26].

Materials and Methods

Male Sprague-Dawley rats (150–200 g) inbred at Süddeutsches Tierzuchtinstitut, Tuttlingen, F.R.G. (SUT : SDT) were used throughout this study. The animals were fasted overnight (14–18 h) with free access to water before they were killed by decapitation.

Procedure A: Subcellular fractionation in the prescence of cations. Isolation of intact mitochondria-RER complexes

Livers were perfused in situ with ice-cold 0.9% NaCl, cut into small pieces and these were washed once in cold 1.15% potassium chloride. All further isolation steps were done in the cold room at 0–4%. After determination of wet weight, a 1 : 4 homogenate was prepared in 0.35 M sucrose containing 2.5 mM magnesium acetate and 10 mM Tris acetate, pH 7.4 (designated 'buffer 1'). Homogenisation was performed in a 60 ml glass-Teflon Potter-Elvejhem homogenizer (radial clearance 0.18–0.24 mm) rotating at 900 rev./min (11 strokes). The homogenate was filtered through two layers of sterile gauze and once more through one layer of Miracloth (Calbiochem, Lucerne, Switzerland). The general fractionation scheme and the subfractionation of total microsomes into rough and smooth microsomes is outlined in Fig. 1. The mitochondrial rough endoplasmic reticulum complex fraction (MITO-RER) was prepared by a modification of the technique described by Lewis and Tata (Ref. 23, Fig. 1). The crude nuclear fraction was resuspended in 3 vol. of buffer 1 in a Dounce homogenizer (loose fitting pestle, 5 strokes). 12.5 ml of this suspension were diluted with

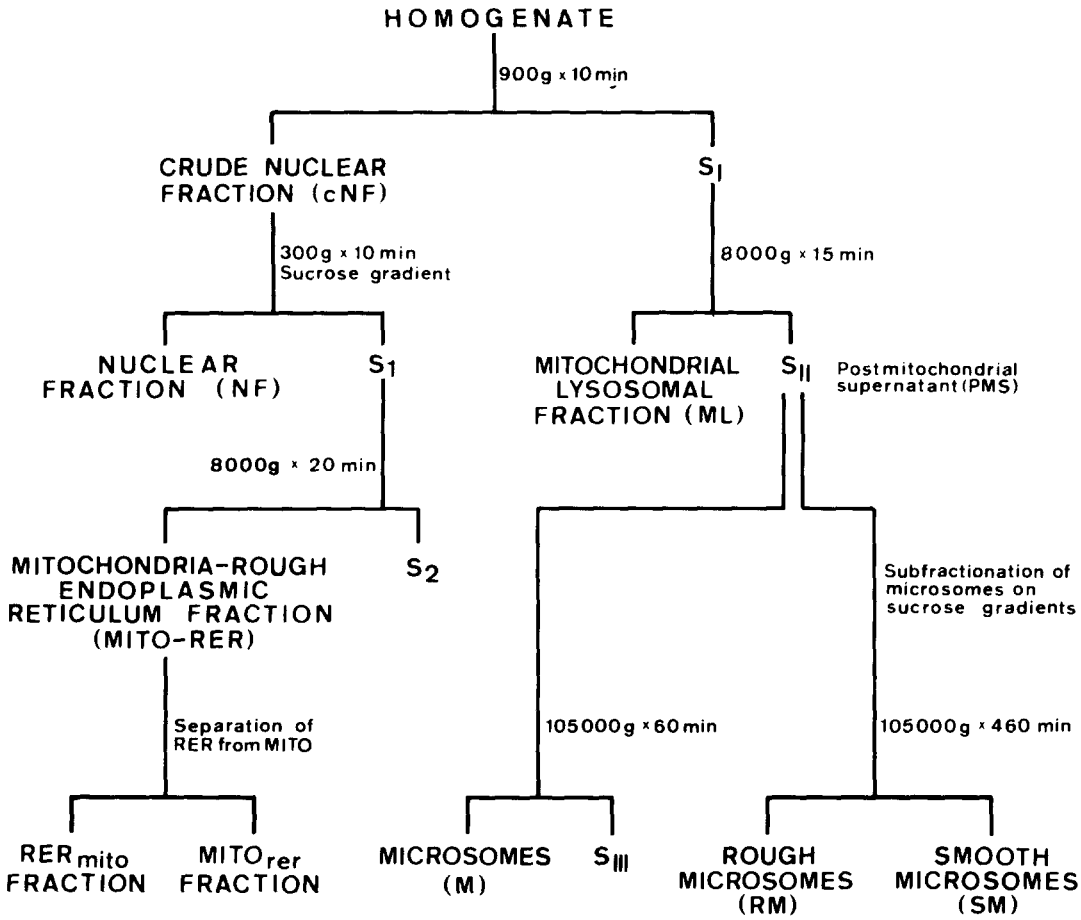

Fig. 1. Schematic representation of subcellular fractionation according to procedure A. The homogenate was first centrifuged at 900 X g for 10 min to sediment the crude nuclear fraction (cNF). From the supernatant, the mitochondrial-lysosomal fraction (ML) was pelleted at 8000 X g for 15 min and the microsomes (M) sedimented from the postmitochondrial supernatant at 105 000 X g for 60 min (MSE Superspeed 50 ultracentrifuge, fixed angle rotor No. 59118). Microsomes were further subfractionated into rough (RM) and smooth (SM) microsomes by layering 8 ml of undiluted postmitochondrial supernatant over a two-step discontinuous gradient consisting of 3 ml of 1.3 M and 1 ml of 0.6 M sucrose [14] and centrifuged at 105 000 X g for 7 h and 40 min [28] in a Beckman 50 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). SM, which banded at the interface of the 0.6 M sucrose to the 1.3 M sucrose layer and the pelleted RM were recovered as described in detail by Dallner [26]. Control parameter for successful subfractionation was a RNA to protein ratio of approx. 0.25 for RM and <0.07 for SM. The exact isolation procedure of MITO-RER complexes and the purification of the mitochondria associated RER (RER_{mito}) are described in the text.

6.0 ml cold water and 7.5 ml buffer 1 to give a sucrose concentration of 0.27 M. 13 ml of the diluted crude nuclear fraction were carefully underlayered with 15 ml of buffer 1 and centrifuged for 10 min in a 30 ml tube at 1050 rev./min (MSE Mistral 4L, swing-out rotor No. 62303). From each tube, 23 ml of the opaque supernatant were aspirated and the suspended material (S_1) concentrated by centrifugation at 8000 X g for 20 min to yield the MITO-RER fraction. The methods used for separation of RER (RER_{mito}) from mitochondrial components ($MITO_{rer}$) of the MITO-RER complex fractions are described in legends to Table III and Table IV, respectively.

Procedure B: Subcellular fractionation without use of exogenous cations

In this procedure, the fractions M, RM and SM were isolated in the absence of any added cations,

using only sucrose solutions during the entire fractionation procedure. The various centrifugation steps were similar to those in procedure A, however, for the sake of purity and to prevent aggregation of membranes during subfractionation, minor modifications had to be introduced (for comparison see legend to (Fig. 1). Livers were perfused in situ with cold 0.25 M sucrose via the portal vein and cut into small pieces. These were washed in 0.25 M sucrose and a 1 : 4 homogenate prepared in 0.35 M sucrose. The postmitochondrial supernatant was prepared by centrifuging the initial 900 × *g* supernatant (S_1) at 10 000 × *g* for 20 min [26]. For subfractionation into rough microsomes and smooth microsomes, the postmitochondrial supernatant was diluted with 0.35 M sucrose to a volume of 5 ml per 1 g of liver wet weight, layered over the same two step gradient of sucrose (0.6 M and 1.3 M) and centrifuged as described under procedure A.

Isopycnic centrifugation of rough microsomes on linear metrizamide gradients

Rough microsomes were isolated either under the ionic conditions described in procedure A or in cation-freesucrose solutions (procedure B). Freshly prepared rough microsomes were resuspended in 0.25 M sucrose to a protein concentration of 8–12 mg/ml and 1 ml of this suspension layered over a linear gradient of 22–44% metrizamide. The gradients were centrifuged at 40 000 rev./min for 6 h in a Beckman SW 41 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). 1 ml fractions were collected through the bottom of the tube and densities determined from measured refractive indices. Since microsomal vesicles interfere with the estimation of refractive index, tubes without microsomes were centrifuged simultaneously [30].

Biochemical methods

All subcellular fractions isolated either in the presence or absence of cations were resuspended in 0.25 M sucrose (10–20 mg protein/ml) and stored frozen (–20°C) for a maximum of 48 h before they were assayed. Once freezing and thawing did not alter enzyme activities significantly.

Glucose-6-phosphatase was assayed in the presence and absence of 0.03% deoxycholate according to the method of Baginsky et al. [31] using aliquots of samples of 100–400 μg protein in a total assay volume of 0.4 ml [30,32]. Activity is expressed as μmol P_i liberated per min.

Cytochrome oxidase was directly determined by following the oxidation of reduced cytochrome *c* at 550 nm in relation to 541 nm (dual-wave-length mode of a DW-2 Spectrophotometer, American Instrument Co., Silver Springs. MD) using a millimolar extinction coefficient of 21 [33]. Reduced cytochrome *c* (Sigma Type IV) was prepared as described by Wharton and Tzagaloff [34]. Data are expressed as μmol cytochrome *c* oxidized per min.

Cytochrome P-450 was estimated in subcellular fractions by the $(CO + Na_2S_2O_4) - (Na_2S_2O_4)$ difference spectrum [35] using an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ for the difference in absorption between 450 and 490 nm. In the MITO-RER fraction the absorbance change at 460 nm relative to 490 nm was used since this wave-length pair was not influenced by the amount of cytochrome oxidase present [36]. The extinction coefficient for this wavelength pair was evaluated in a special set of experiments where various amounts of rat heart mitochondria were mixed with liver microsomes and was found to be 36.4 $mM^{-1} \cdot cm^{-1}$.

Cytochrome b_5 was measured according to Omura and Sato [35].

Protein was estimated by the method of Lowry et al. [37] using bovine serum albumin as standard.

RNA was estimated by the method described by Fleck and Munroe [38].

Electron microscopy

Freshly excised liver tissue was minced and immediately immersed in 1% osmium tetroxide buffered to pH 7.4 with 0.1 M *s*-collidine containing 0.22 M sucrose at 4°C. After a fixation period of 4 h, the tissue was washed in 0.05 M maleate buffered with NaOH to pH 5.2 for 45 min at 4°C, block-stained with 0.5% uranylacetate and again washed for 30 min in the same maleate buffer [39]. Membrane preparations were pelleted, resuspended in 0.25 M sucrose and fixed in suspension with 2.5% glutaraldehyde containing 0.1 M sodium cacodylate (pH 7.4) and 0.17 mM calcium chloride for 2 h.

The suspended material was then centrifuged (8000 × *g* for 20 min for MITO-RER complexes, 105 000 × *g* for 60 min for microsomal subfractions).

Pellets were diced, briefly washed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.17 mM $CaCl_2$ and postfixed for 2 h in 1% osmium tetroxide/0.1 M sodium cacodylate (pH 7.4) at 4°C. Liver tissue blocks and fixed subcellular fractions were dehydrated in a graded series of ethanol (70–100%) and propylenoxide for 3–4 h and embedded in Epon. Sections were stained with 1% uranylacetate and alkaline lead citrate [40] and examined in a Philips EM model 201 or 200.

Chemicals

Chemicals for electron microscopy were from Polysciences, Inc., Warrington, PA. U.S.A., metrizamide from Nyegaard Co. A/S, Oslo, Norway. All other chemicals used in this study were of analytical grade and were purchased from either BDH Chemicals Ltd., Poole, U.K.; Fluka AG, Buchs, Switzerland; Merck, Darmstadt, F.R.G., or Sigma Chemical Co., St. Louis, MO, U.S.A.

Results and Discussion

Distribution of endoplasmic reticulum membranes and mitochondria between subcellular fractions prepared in the presence of exogenous cations. Isolation of intact MITO-RER complexes

Perfusion of rat livers with isotonic NaCl followed by fractionation in the presence of exogenous cations in the form of 10 mM Tris-acetate buffer (pH 7.4) and 2.5 mM magnesium acetate caused 40–50% of fragments of endoplasmic reticulum, mostly representing RER (44% of RNA), to sediment together with the majority of mitochondria (65% of cytochrome oxidase) already during the first low speed centrifugation step (crude nuclear fraction, Table I). Consequently, the yield of microsomes isolated from the postmitochondrial supernatant was reduced to 14 mg protein per g liver (wet weight), which is in agreement with reported yields of microsomes prepared with cations-containing media [41,42].

TABLE I

DISTRIBUTION OF PROTEIN, RNA AND MARKER ENZYME ACTIVITIES FOR ENDOPLASMIC RETICULUM AND MITOCHONDRIA BETWEEN VARIOUS SUBCELLULAR FRACTIONS OBTAINED FROM RAT LIVERS PERFUSED WITH 0.9% NaCl

Subcellular fractions were prepared as described under Materials and Methods, procedure A, and as outlined in Fig. 1. For homogenates data are expressed per g wet weight. Cytochrome *c* oxidase and glucose-6-phosphatase activity (U) is expressed as μmol cytochrome *c* oxidized and as μmol P_i liberated per min, respectively. For subcellular fractions % is based on homogenate representing 100%. Total recovery refers to the fractions cNF, ML, M and cell sap (S_{III}). Data are given as the mean ±S.D. with the number of experiments indicated in parenthesis.

	Protein (16)	RNA (10)	Cytochrome *c* oxidase (7)	Glucose-6-phosphatase (14)	Cytochrome *P*-450 (12)
Homogenate	mg/g liver	mg/g liver	U/g liver	U/g liver	nmol/g liver
	125 ± 14	5.8 ± 0.6	5.5 ± 1.5	10.7 ± 1.7	20.0 ± 2.4
Subcellular fractions	%	%	%	%	%
cNF	48.3 ± 4.7	43.7 ± 5.3	64.6 ± 9.8	44.5 ± 2.8	38.0 ± 5.3
NF	16.2 ± 4.9	18.6 ± 3.2	14.4 ± 3.3	16.8 ± 3.0	15.1 ± 2.4
MITO-RER	22.8 ± 4.5	19.8 ± 3.5	46.5 ± 5.4	20.5 ± 3.5	13.4 ± 2.0
ML	12.2 ± 2.2	7.1 ± 3.5	21.3 ± 5.1	7.1 ± 2.7	10.3 ± 2.9
M	11.3 ± 1.7	33.4 ± 3.7	1.8 ± 0.8	36.8 ± 8.0	48.7 ± 7.3
S_{III}	26.0 ± 1.6	12.6 ± 2.0	–	3.5 ± 1.2	–
Recovery	97.8%	96.8%	87.7%	92.0%	97.0%

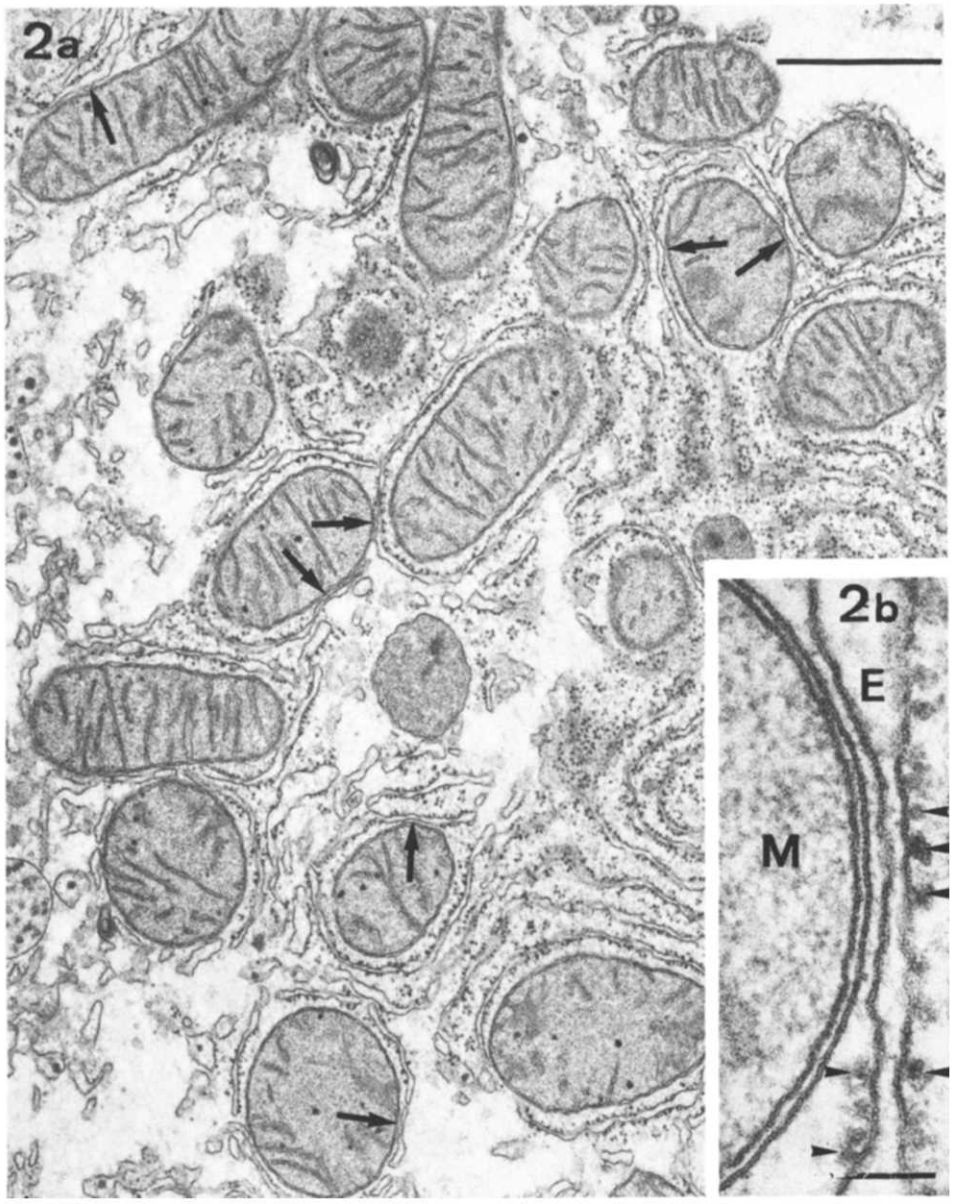

Fig. 2. Electron micrograph of normal rat liver in vivo. (a) Section of normal rat liver showing a number of mitochondria associated with cisternae of RER. Arrows point to areas of close appositions between smooth regions of RER and outer mitochondrial membrane. Bar 1.0 μm. (b) High power micrograph of a site of close association between RER and mitochondrion. Note that the ER membrane is agranular over the area of close apposition. M, mitochondrion. E, endoplasmic reticulum. Arrow heads point to ribosomes. Bar 0.1 μm.

Further subfractionation of the resuspended crude nuclear fraction (cNF) (Fig. 1) resulted in a recovery of 47% of mitochondria and 10% of RER in the MITO-RER fraction (Fig. 3a, Table I). This fraction was virtually devoid of nuclei and larger cell debris as judged by careful microscopical control of semithin sections of the whole pellet. However, a significant loss of mitochondria and RER into the nuclear

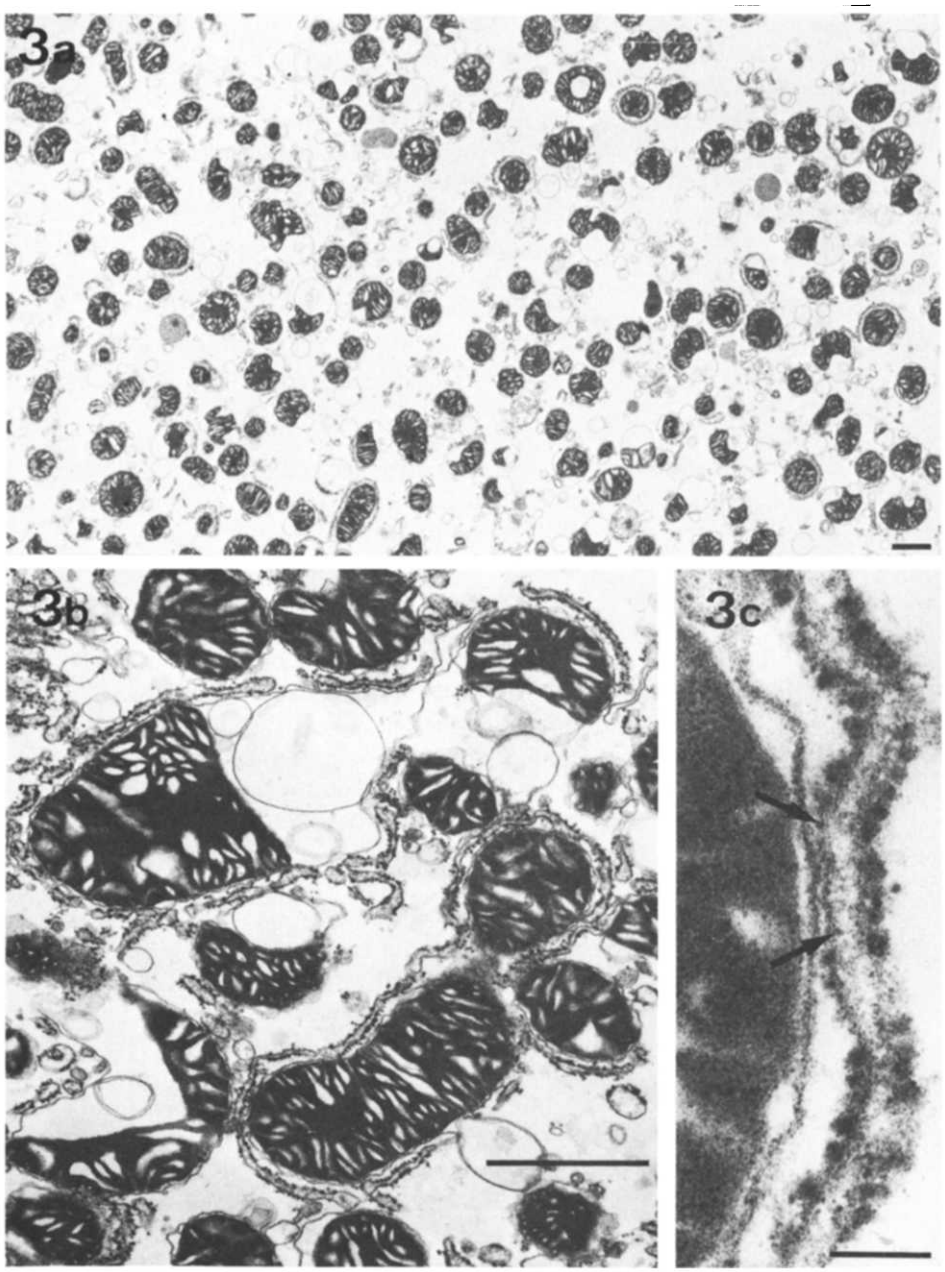

Fig. 3. Electron micrograph of isolated MITO-RER fraction. (a) Low-power electron micrograph of isolated MITO-RER fraction. Bar 1.0 μm. (b) Higher magnification of isolated MITO-RER complexes showing most of mitochondria partially surrounded by single cisternae of RER. Bar 1.0 μm. (c) High-power micrograph of a site of close association between RER and mitochondrion in an isolated organelle complex. The portion of RER which is closely apposed to the mitochondrion appears free of ribosomes (arrows). Bar 0.1 μm.

fraction (NF) had to be accepted. An additional loss of 5–10% of RNA and of glucose-6-phosphatase activity occurred during the sedimentation of MITO-RER complexes at 8000 × *g*, probably reflecting vesiculated fragments of RER and SER in the crude nuclear fraction.

The MITO-RER fraction is characterized by the presence of mitochondria predominantly in the condensed state which frequently are associated with single cisternae of RER (Figs. 3a, b). These isolated organelle complexes show a striking similarity to the associations between RER and mitochondria commonly seen in intact liver cells (Fig. 2a). Both in vitro and in vivo associated RER cisternae frequently exhibit areas of close appositions to the outer mitochondrial membrane. At these sites the ER membranes regularly appear free of ribosomes, leaving an intermembranous space of surprisingly constant width (Figs. 2b, 3c).

Influence of cations on sedimentation of endoplasmic reticulum membranes and on the composition of the microsomal fraction

Initial perfusion of the liver with NaCl has marked effects on the distribution pattern of glucose-6-phosphatase and RNA between subcellular fractions, even if all subsequent fractionation steps are done in relative cation-free sucrose (Fig. 4). Thus, when compared to an initial perfusion with isotonic sucrose, NaCl perfusion caused an increase in the sedimentation of RER membranes into the crude nuclear fraction of 30 to 40%, while the amount of endoplasmic reticulum membranes in the mitochondrial lysosomal (ML) fraction remained virtually unchanged. The increase in early sedimentation of RER membranes after NaCl perfusion was accompanied by the cosedimentation of approx. 60% of mitochondria (data not shown). Ultrastructural examination of the cNF isolated after initial NaCl perfusion with all subsequent steps in magnesium-free 0.35 M sucrose revealed still frequent intimate contacts between mitochondria and RER, which under these conditions was found mostly in the form of vesiculated fragments (pictures not shown). Thus, the presence of magnesium ions (2.5 mM) apparently was required to stabilize the cisternal structure of RER associated with mitochondria.

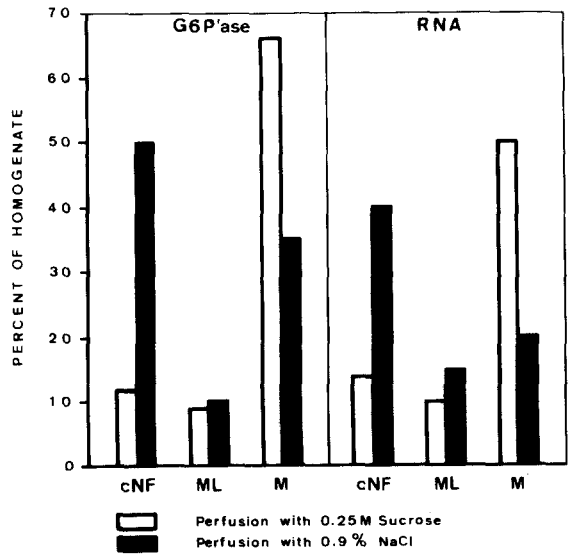

Fig. 4. Effect of perfusion of liver with NaCl versus sucrose on the distribution of glucose-6-phosphatase and RNA in different subcellular fractions. Homogenates (1 : 4) from sucrose- or NaCl-perfused livers were subfractionated by differential centrifugation according to procedure B.

The influence of the presence of exogenous cations on the final recovery and enzymatic composition of the microsomal fraction (M) as well as its rough (RM) and smooth (SM) subfractions is summarized in Table II. Using exclusively cation-free sucrose solutions during subcellular fractionation (procedure B), about 20 mg of microsomal protein per g liver could be recovered from the initial post-mitochondrial supernatant, which is in agreement with values reported by others [14,16,18,43]. Initial NaCl perfusion of livers lowered the protein recovery of total microsomes by approx. 50%, which could be fully accounted for by a decrease in the yield of rough microsomes. In contrast, the recovery of smooth microsomes was reduced only if magnesium ions were present in the sucrose medium. Mg^{2+} had no additional effect on the recovery of rough microsomes.

Subcellular fractionation in the presence and absence of exogenous cations resulted in rough microsomal subfractions of apparently different enzymatic composition (Table II). Rough microsomes isolated with sucrose alone (Procedure B) showed a strikingly higher glucose-6-phosphatase activity and a slightly increased cytochrome *P*-450 concentration when compared to rough microsomes isolated from a post-mitochondrial supernatant according to Procedure A.

TABLE II

EFFECT OF CATIONS ON RECOVERY AND ENZYMATIC COMPOSITION OF MICROSOMES (M) AND THEIR ROUGH (RM) AND SMOOTH (SM) SUBFRACTIONS

Fractions were isolated according to procedure A (presence of cations) or procedure B (cation-free sucrose) as described under Materials and Methods. In procedure C, the liver was perfused with cold 0.9% NaCl as in procedure A, but the subsequent fractionation procedure was done in cation-free sucrose, as in procedure B. Data are expressed as mean ± S.D. (number of experiments in parenthesis) except for procedure C, where mean ± range is given. Student's *t*-test was used for calculation of *P* values. n.s., not significant.

		M	*P*	RM	*P*	SM	*P*
Recovery protein (mg/g liver)							
	Procedure A (11)	8.1 ± 1.1		3.8 ± 0.8		3.6 ± 0.9	
	Procedure B (8)	19.0 ± 1.9		8.7 ± 1.4		4.9 ± 0.5	
	Procedure C (2)	9.4 ± 1.3		3.9 ± 0.1		4.9 ± 0.1	
Concentration or activity							
RNA (mg/mg protein)	A (9)	0.154 ± 0.01	⩽0.05	0.246 ± 0.028	n.s.	0.059 ± 0.012	n.s.
	B (3)	0.129 ± 0.010		0.232 ± 0.020		0.050 ± 0.010	
Glucose-6-phosphatase (μmol P_i/mg protein per min)	A (9)	0.291 ± 0.032	⩽0.01	0.291 ± 0.021	⩽0.001	0.318 ± 0.050	n.s.
	B (5)	0.351 ± 0.040		0.389 ± 0.026		0.327 ± 0.039	
Cytochrome *P*-450 (nmol/mg protein)	A (7)	0.713 ± 0.063	n.s.	0.430 ± 0.052	⩽0.010	0.838 ± 0.150	n.s.
	B (11)	0.706 ± 0.056		0.518 ± 0.052		0.801 ± 0.152	
Cytochrome b_5 (nmol/mg protein)	A (9)	0.338 ± 0.030	n.s.	0.184 ± 0.020	n.s.	0.405 ± 0.084	n.s.
	B (7)	0.308 ± 0.031		0.203 ± 0.022		0.383 ± 0.077	

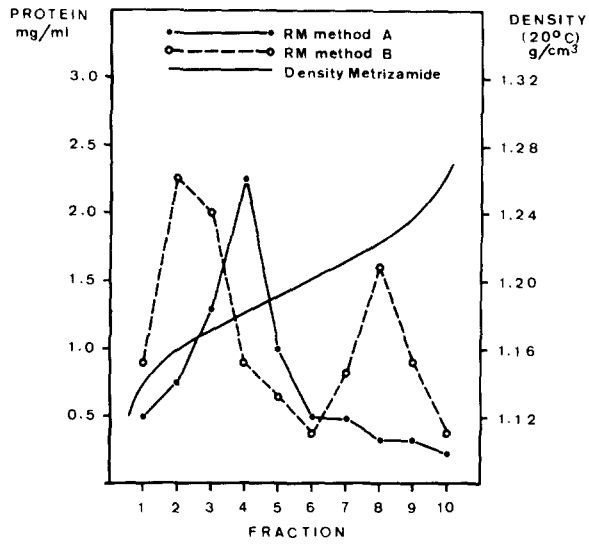

Fig. 5. Equilibrium density of rough microsomes in metrizamide density gradient. 8–12 mg protein of RM isolated either in the presence of cations (procedure A) or in cation-free sucrose solutions (procedure B) were layered over a linear gradient of 22–44% metrizamide and centrifuged at 284 000 × *g* for 6 h. Fractions were then collected through the bottom of the tubes (fraction 1 = top of gradient; fraction 10 = bottom of gradient) and analyzed for protein content. Densities were determined from refractive indices measured on samples from tubes centrifuged simultaneously without microsomal material.

In contrast to rough microsomal subfractions, smooth microsomes isolated in the presence and absence of exogenous cations had the same glucose-6-phosphatase activity and cytochrome *P*-450 concentrations per mg protein. Independent of the isolation procedure, the cytochrome *P*-450 and cytochrome b_5 concentrations per mg of protein were 1.5- to 2.0-fold higher in smooth microsomes than in rough microsomes, in accordance with observations by Gram et al. [44,45]. These data suggested that a distinct subtype of RER membranes may be associated with MITO-RER complexes. Additional evidence for this concept was provided in experiments in which rough microsomes were isolated in cation-free sucrose (procedure B) and subjected to centrifugation in linear metrizamide gradients. In accordance with the report of Fehrström et al. [30], the membranes were distributed in two major groups with mean densities of 1.16 g/cm^3 and 1.21 g/cm^3, respectively (Fig. 5). The activity of glucose-6-phosphatase was found to be 0.415 in the heavier and 0.293 μmol P_i/mg protein per min in the lighter subfractions of rough microsomes. In contrast, rough microsomes isolated from NaCl-perfused livers and in the presence of 2.5 mM magnesium acetate (procedure A) yielded only one band at a density of about 1.18 g/cm^3 exhibiting an activity of glucose-6-phosphatase of 0.275. The small shift to higher density from 1.16 to 1.18 g/cm^3 could be due to slight aggregations which regularly occurred during centrifugation of 'cations-isolated' rough microsomes in metrizamide gradients. These data strongly suggest that the high density, glucose-6-phosphatase enriched subfraction of 'sucrose rough microsomes' corresponds to mitochondria-associated RER, which sediments at low centrifugal force in the presence of cations because of their intact associations with mitochondria.

Separation and characterization of RER (RER_{mito}) from MITO-RER complexes

In order to directly evaluate the specific activity of glucose-6-phosphatase in mitochondria associated RER, we tried to separate RER from mitochondria. This was first attempted with small concentrations of EDTA. Thus, whereas in the absence of EDTA only about 12% of endoplasmic reticulum membranes could be separated from adhering mitochondria, rehomogenisation of the MITO-RER fraction in the presence of 0.5 mM EDTA increased the recovery of dissociated endoplasmic reticulum membranes to around 50% (Table III). Glucose-6-phosphatase was higher in RER_{mito} as compared to rough microsomes isolated from the corresponding postmitochondrial supernatant (Table II, Procedure B). Increasing the EDTA concentration to 25 mM decreased the recovery of RER_{mito} drastically although ribosomes were further released from endoplasmic reticulum (data not shown). Because of this marked detachment of ribosomes from endoplasmic reticulum membranes (recovery of RNA was decreased to 42% at 0.5 mM EDTA, Table III) and because EDTA also may affect glucose-6-phosphatase activity, isolation of RER_{mito} was additionally attempted in the absence of EDTA. The data in Table IV indicate that after swelling and contraction of mitochondria by hypotonic/hypertonic conditions 47% of RNA and 70% of glucose-6-phosphatase of the original MITO-RER fraction could be recovered as RER_{mito}. Based on the

TABLE III

EFFECT OF HYPOTONIC/HYPERTONIC TREATMENT ON THE RELEASE OF RER (RER_{mito}) FROM MITO-RER COMPLEXES

The MITO-RER fraction isolated from 5 g liver (wet weight) was resuspended in 20 ml of either 0.25 M sucrose or 0.25 M sucrose containing 0.5 mM EDTA (pH adjusted to 7.6 with Tris-HCl buffer) to yield a suspension of 4–5 mg protein per ml. This suspension was rehomogenized at 800 rev./min (radial clearance 0.18–0.24 mm, eight up and down strokes) and centrifuged at 8000 × *g* for 10 min to sediment the mitochondria partially stripped from adhering RER, named MITOrer. The rough microsomal membranes remaining in the supernatant were collected by centrifugation at 105 000 × *g* for 60 min and were designated RERmito. Data are the mean of two experiments.

Treatment	Subcellular fraction	RNA		Glucose-6-phosphatase		Cytochrome oxidase (μmol cycochrome *c*/mg protein/min)
		mg/g liver	mg/mg protein	μmol P_i/g liver/min	μmol P_i/mg protein/min	
–	MITO-RER	0.94	0.050	1.85	0.092	0.143
0.25 M sucrose	MITOrer	0.63	0.048	1.25	0.087	–
(Control)	RERmito	0.16	0.148	0.22	0.210	0.052
0.25 M sucrose +	MITOrer	0.26	0.030	0.71	0.061	–
0.5 mM EDTA	RERmito	0.14	0.149	0.93	0.528	0.031

specific activity of cytochrome oxidase, this RER_{mito} fraction was 1.8-times more contaminated with inner mitochondrial membrane than the corresponding rough microsomes. The RER_{mito} subfraction also contained fragments of the outer mitochondrial membranes. However, these contaminations did not prevent the expression of a higher specific glucose-6-phosphatase activity in RER_{mito} as compared to the corresponding rough microsomes (Tables II, IV). Morphologically RER_{mito}, either isolated by EDTA or by hypotonic/hypertonic treatment of intact MITO-RER complexes, was uniquely present as vesiculated particles, i.e. in the familiar structure of rough microsomes isolated from the postmitochondrial supernatant (Fig. 6).

The differences in glucose-6-phosphatase activities

TABLE IV

EFFECT OF HYPOTONIC/HYPERTONIC TREATMENT ON THE RELEASE OF RER (RER_{mito}) FROM MITO-RER COMPLEXES

The isolated MITO-RER complexes were resuspended in hypotonic 20 mM Tris-phosphate buffer (pH 7.8) to a protein concentration of 5–10 mg/ml and mitochondria were allowed to swell for 10 min [29]. After addition of 1/4 vol. of 2.0 M sucrose and stirring for another 10 min the suspension was carefully rehomogenized in a Potter-Elvejhem glass Teflon homogenizer (600 rev./min radial clearance 0.18–0.24 mm, four up and down strokes). The inner mitochondrial membranes were sedimented at 800 × *g* for 8 min and the vesiculated ER contained in the first and second 8000 × *g* supernatants combined and pelleted at 105 000 × *g* for 60 min. This fraction is also denoted RERmito (Fig. 1). Because outer mitochondrial membranes are known to be virtually free of glucose-6-phosphatase, this fraction was not further purified. Data are expressed as the mean ± S.D. of six experiments.

Treatment	Subcellular fraction	RNA		Glucose-6-phosphatase		Cytochrome oxidase (μmol cytochrome *c*/mg protein/min)
		mg/g liver	mg/mg protein	μmol P_i/g liver/min	μmol P_i/mg protein/min	
	MITO-RER	1.10 ± 0.30	0.052 ± 0.009	2.20 ± 0.40	0.098 ± 0.018	0.155 ± 0.025
20 mM Tris-phosphate followed by 2.0 M sucrose	MITOrer	0.23 ± 0.06	0.021 ± 0.002	0.45 ± 0.05	0.037 ± 0.005	–
	RERmito	0.52 ± 0.03	0.145 ± 0.005	1.50 ± 0.27	0.451 ± 0.024	0.027 ± 0.004

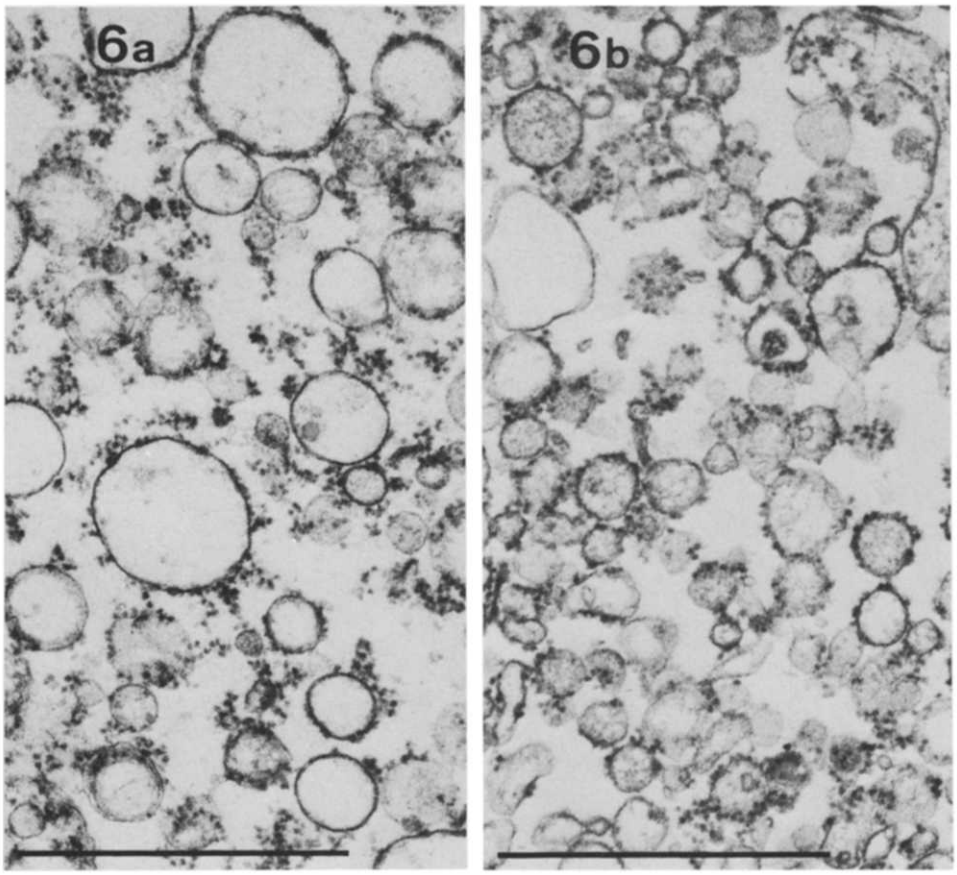

Fig. 6. Electron micrograph of two subfractions of RER isolated by different procedures. (a) Rough microsomes prepared from postmitochondrial supernatant according to procedure A, as described in Methods. (b) RER membranes (RER_{mito}) separated from MITO-RER complexes by means of hypotonic/hypertonic treatment as described in legend to Table IV. The separation procedure resulted in the vesiculation of the RER cisternae. Bar 1.0 μm.

between RER_{mito} and rough microsomes persisted even after detergent activation of the glycolytic enzyme by 0.03% deoxycholate (Table V). These data demonstrate that the heterogeneity in the distribution of glucose-6-phosphatase between the two rough subfractions of endoplasmic reticulum most likely is due to different enzyme concentrations rather than to an inequality in the physico-chemical state of membranes [32]. In contrast to rough microsomes, smooth microsomes were highly activated by deoxycholate to the same level of glucose-6-phosphatase activity as RER_{mito}. These results confirm that with respect to glucose-6-phosphatase a unique part of RER can be isolated in close association with mitochondria from rat liver. It was not possible to directly demonstrate that this RER_{mito} indeed corresponds to the high density subfraction of rough microsomes isolated in cation-free sucrose (as suggested from data in Fig. 5) because the density of RER_{mito} was lowered by the loss of membrane-bound ribosomes during the isolation procedure (Table III, IV) and consequently banded at a mean density of 1.18 g/cm^3 during isopycnic centrifugation in metrizamide gradients (data not shown).

General discussion

This study confirms that a sizeable portion of hepatic endoplasmic reticulum can be recovered in close association with mitochondria from low speed

TABLE V

GLUCOSE-6-PHOSPHATASE ACTIVITY OF ENDOPLASMIC RETICULUM MEMBRANES ISOLATED FROM MITO-RER COMPLEXES AND FROM POSTMITOCHONDRIAL SUPERNATANT IN THE PRESENCE AND ABSENCE OF 0.03% DEOXYCHOLATE (DOC)

The various subfractions of endoplasmic reticulum were isolated according to procedure A as described in Materials and Methods. RERmito were prepared from the MITO-RER fraction by hypotonic/hypertonic treatment (see legend to Table IV). Data are given as the mean ± S.D. of six experiments and were compared by Student's *t*-test.

	Glucose-6-phosphatase (μmol P_i/mg protein/min)	
	−DOC	+DOC
RER_{mito}	0.451 ± 0.024 [a]	0.504 ± 0.027 [b]
Rough microsomes	0.302 ± 0.023	0.332 ± 0.028
Smooth microsomes	0.343 ± 0.037	0.512 ± 0.027

[a] Different from RM and SM ($P < 0.001$).
[b] Different from RM ($P < 0.001$).

sediments of liver homogenates prepared under defined ionic conditions. This rapidly sedimenting endoplasmic reticulum apparently corresponds to the high-density subfraction of rough microsomes isolated in the presence of cation-free isotonic sucrose according to well established fractionation schemes (Fig. 5; Ref. 26). Endoplasmic reticulum associated with mitochondria had a higher content of glucose-6-phosphatase than endoplasmic reticulum contained in the corresponding postmitochondrial supernatant. The biochemical and morphological resemblance of this subfraction of endoplasmic reticulum to RER and the apparent structural association with mitochondria lead us to propose the term mitochondria-rough endoplasmic reticulum (MITO-RER) complexes despite the fact that patches of smooth endoplasmic reticulum also occur in this fraction, particularly at sites of close appositions of endoplasmic reticulum with mitochondria. These data support the concept that heterogeneities in enzyme activities observed in subfractions of isolated endoplasmic reticulum membranes may indeed reflect intracellular heterogeneity of endoplasmic reticulum rather than differences in the physical property of these membranes in vitro [17]. However, whether this is true to the same extent for all hepatocytes within the hepatic lobule cannot be decided from this study, because we do not known whether we recovered MITO-RER complexes in equal number from peripheral, midzonal and centrilobular hepatocytes [9].

The morphological structure of the isolated MITO-RER complexes closely resembles similar associations commonly seen in intact liver (Fig. 2b, 3c). Thread-like connections and even direct membranous continuities between endoplasmic reticulum and outer mitochondrial membranes have been observed in various plant and animal tissues, including rat liver [46,47]. We have no direct evidence for membranes being physically continuous between the two organelles in the present study. Interestingly however, and in agreement with observations reported by other investigators [22,25], it was extremely difficult to separate RER_{mito} from mitochondria by either mechanical or other means, e.g. EDTA or hypotonic/hypertonic treatment (Table III, IV).

In contrast to the rapidly sedimenting endoplasmic reticulum fraction which previously has been isolated by differential centrifugation from unperfused rat liver by Lewis and Tata [23], we found no clusters of bilamellar RER in the MITO-RER fraction (Fig. 3a). Only 20% of total glucose-6-phosphatase was recovered in our MITO-RER fraction (Table I) instead of 30% by Shore and Tata [22]. Thus, initial perfusion of the liver with NaCl may predominantly affect that portion of RER which is present in the form of bilamellar stacks in intact hepatocytes. Its dissociation and consequent transformation into vesiculated particles during homogenisation could then result in its sedimentation as rough microsomes from the postmitochondrial supernatant. Alternatively, NaCl may selectively protect the in vivo occurring associations between single cisternae of RER and mitochondria and therefore cause the cosedimentation of the two organelles into low-speed pellets (Fig. 4). This effect appears not to be mediated by sodium induced ribosomal interaction with outer mitochondrial membranes, because (a) areas of close appositions of RER to mitochondria appear free of ribosomes (Fig. 3c) and (b) stripping of ribosomes from RER_{mito} either by EDTA alone (concentrations higher than 5 mM in this study; data not shown) or potassium chloride and EDTA did not release endoplasmic reticulum membranes from mitochondria of

MITO-RER complexes [25]. Interestingly, although Pickett et al. [25] isolated MITO-RER by rate zonal centrifugation in a linear sucrose gradient, livers also were initially washed in ice-cold saline supporting the suggested important initial role of monovalent cations for isolation of intact MITO-RER complexes.

The cations required for in vitro protection of MITO-RER complexes markedly influenced the yield and enzymatic composition of rough microsomes isolated from the postmitochondrial supernatant when compared to rough microsomes prepared in the absence of exogenous cations (Table II). Several studies in which the rough components of sucrose-isolated microsomes were further subfractionated revealed an apparent accumulation of glucose-6-phosphatase in high-density rough microsomes [12,13,15, 27], i.e. in vesicles with a large number of bound ribosomes [48]. The present study demonstrates that the mitochondria-associated RER most likely is identical with this high density and glucose-6-phosphatase enriched subfraction of rough microsomes (Fig. 5, Table V). This interpretation appears to be valid even under the assumption of possible contaminations of isolated subfractions with Golgi membranes, since only a very small amount of glucose-6-phosphatase activity has been found in association with this organelle [49,50].

One of the general conclusions which can be made on the basis of the present study is that inclusion of monovalent cations into isolation media leads to a sorting out of RER membranes during subcellular fractionation of normal rat liver tissue. This may be the case even with the use of weakly buffered sucrose solutions for preparation of homogenates from unperfused rat liver [17]. On the other hand, our findings confirm that at least qualitatively rough microsomes contained in the postmitochondrial supernatant prepared with the exclusive use of cation-free isotonic sucrose are more representative of the spectrum of all RER membranes occuring in intact liver [14–16,19,26].

It is impossible, of course, to unequivocally rule out that the isolated organelle complexes represent the artificial result of the isolation procedure since neither in vivo nor in vitro we have specific markers for MITO-RER complexes. However, the morphological and biochemical data presented in this study as well as the extensive centrifugation data provided by Shore and Tata [22] and Pickett et al. [25] do not support the interpretation of artifactual generation of MITO-RER complexes. Furthermore, the observations that the isolated organelle complexes may serve specific functions during assembly of the microsomal hemoprotein cytochrome *P*-450 [24,51,52] also suggest that the associations between RER and mitochondria in vivo may not occur at random. However, the exact role of MITO-RER complexes during biosynthesis of cytochrome *P*-450 and other hemoproteins has yet to be elucidated. This is presently attempted in our laboratory by combining selective isolation of MITO-RER complexes and specific detection of newly formed apocytochrome *P*-450 by immunochemical techniques.

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