

INTEGRATED STEREOLOGICAL AND BIOCHEMICAL STUDIES ON HEPATOCYtic MEMBRANES

I. Membrane Recoveries in Subcellular Fractions

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ABSTRACT

Previous attempts to relate the structure and function of hepatocytic membranes have compared biochemical data of fractions to morphological data derived from either intact tissue or fractions. The effects of the original homogenization aside, biochemical recoveries comparing membrane marker enzymes of the homogenate to subsequent fractions suggest a general conservation of activity. A stereological study was undertaken to estimate membrane surface areas in the intact tissue, homogenate, and fractions of the same livers and then to test the comparability of these data with membrane marker enzymes by calculating both morphological and biochemical recoveries. The stereological data were corrected for errors due to section thickness and compression.

The average total membrane surface area per 1 g of liver was 9.3 m² in the intact tissue (T), 7.8 m² in the homogenate (H), and 7.4 m² in the fractions (F); recoveries for the membrane surface areas thus averaged 96% for the (F/H) and 81% for the (F/T) comparisons. In homogenate and fractions, the differentiability of membranes by morphological criteria was limited to rough- and smooth-surfaced membranes, as well as outer and inner mitochondrial membranes. The recoveries of rough-surfaced membranes were 101% for F/H and 92% for F/T; those of smooth-surfaced membranes were 89% for F/H and 107% for F/T. For mitochondrial membranes, a recovery of 100% for F/H was obtained, whereas it amounted to only 54% for F/T. With respect to F/H, the membrane recoveries compare well with the marker enzyme recoveries obtained biochemically. The extension of recovery calculations to the intact tissue (F/T) revealed satisfactory conservation of the procedures of homogenization and fractionation; it indicates, however, that a shift of a substantial part of mitochondrial membranes to the pool of unidentifiable smooth membranes may occur on homogenization.

KEY WORDS hepatocytes · morphometry ·
subcellular fractionation · membrane recovery ·
enzyme recovery

Stereological methods have been used to estimate the surface areas of hepatocytic membranes in intact and fractionated livers (3, 4, 7, 21, 27, 35)

and, in some cases, these data have been combined with a biochemical analysis of fractions (17, 30, 36). For example, Stäubli et al. (30) studied concurrent structural and functional changes in hepatocytic membranes associated with phenobarbital induction; they reported parallel increases for the surface area of the endoplasmic reticulum (ER) and for the activities of constituent drug-metabolizing enzymes. In this type of study, however, it is difficult to correlate the morphological and biochemical results because the stereological analysis was performed on the intact tissue, whereas a microsomal fraction—representing only a portion of the original tissue homogenate—was used for the biochemical analysis. The problem arises from the fact that the membranes assayed in a microsomal fraction may or may not faithfully reflect those in the intact tissue. Although Wibo et al. (36) have applied both stereological and biochemical methods to the same microsomal fractions, the relationship of their data to the intact tissue was likewise unknown. In this study, we shall attempt to estimate how well the surface area of membranes of liver cells is conserved throughout homogenization and fractionation procedures.

In biochemistry, the effect of the preparation procedures on specific membranes is determined by calculating recoveries of marker enzymes, which compare the activity of the original material, the homogenate, to the aggregate activities of the resultant fractions. A conservation of activity is generally assumed if the recoveries approach 100%. In analogy to these biochemical procedures, we shall calculate recoveries of membrane surface area estimated by stereological methods. Because of the possibilities to perform a morphometric analysis as well on intact tissue as on fractions and homogenate, it should be possible to extend the calculation of recoveries to include all three preparations, i.e., to estimate how well the membranes found in intact cells are recovered in homogenate and fractions. This paper describes the methods required to collect and analyze the stereological data and presents the recoveries obtained by using analytical cell fractionation on rat liver tissue (1).

MATERIALS AND METHODS

Animals

Adult male albino rats (Sprague-Dawley, Tuttingen, Germany) were fasted for 18 h before decapitation to

deplete glycogen stores; one animal was used for each fractionation. The liver was rapidly removed, a small piece was taken for tissue electron microscopy, and the remainder was immersed in a tared beaker containing ice-cold 0.25 M sucrose (buffered at pH 7.4 with 3 mM imidazole-HCl [Fluka, Buchs, Switzerland]) to determine its weight. The body and liver weights are given in Table I.

Intact Tissue Procedures

ELECTRON MICROSCOPY

Small cubes of tissue were fixed for 10 min at 0°C with 1.5% glutaraldehyde (Taab Laboratories, Reading, England) in a 0.1 M Na-cacodylate buffer (pH 7.4, 340 mosM [adjusted with sucrose]), followed by postfixation for 2 h at 0°C with 1% osmium tetroxide in 0.1 M Na-cacodylate (pH 7.4, 340 mosM [adjusted with NaCl]). The blocks were stained en bloc for 1 h with uranyl acetate (buffered at pH 5.2 in 0.05 M maleate-NaOH buffer) after a 15-min wash in the same buffer (14, 18). After another buffer rinse and dehydration with ethanol, propylene oxide preceded embedding in Epon (24).

All of the electron micrographs to be analyzed stereologically were prepared from thin sections having an interference color of gray to silver. The sections were cut with a diamond knife on a Reichert ultramicrotome (C. Reichert, sold by American Optical Corp., Buffalo, N. Y.) and subsequently expanded with chloroform vapors to reduce compression artifacts. The sections were mounted on 200-mesh grids coated with a carbon-paralodion film and stained with lead citrate (26) for 20 min. Micrographs were recorded on 35-mm film with Philips 200 and 300 microscopes, contact printed, and analyzed in a film projector unit (32) which increases the primary magnification by approximately 10 times. A carbon grating replica with 2,160 lines per mm (Ernest F. Fullam, Inc., Schenectady, N. Y.) was recorded on each film for calibration purposes.

STEREOLOGICAL ANALYSIS OF INTACT TISSUE SAMPLES

The sample micrographs from intact tissue were analyzed stereologically by methods previously described in detail (31–33). To account for the different orders of magnitude of the components investigated, and hence to assure appropriate resolution, the sections were sampled at four stages, for which magnification and reference volume varied (35). For stages II–IV, performed in the electron microscope, 10 sections, each derived from a different block, were used for each animal (Fig. 1).

STAGE I: LIGHT MICROSCOPY, $\times 200$; REFERENCE VOLUME, TOTAL LIVER TISSUE: Volume densities were estimated for the liver parenchyma,¹

¹ Parenchyma is here defined to comprise all hepatocytes, bile canaliculi, sinusoids with their associated cells and spaces, including the space of Disse.

central veins, portal triad areas, branches of the hepatic artery and vein, capsule, and connective tissue of the hilus. Using a cryostat, the right lobe of a rat liver was serially sectioned at 20- μm intervals. Every 1,600 μm , a section was taken and fixed with 4% paraformaldehyde, stained according to van Gieson, and quantitated in an automatic sampling stage microscope (WILD Heerbrugg Inc, Heerbrugg, Switzerland); test points were sampled at 550- μm intervals.

TABLE I
Weight and Volume Reference Systems

Animal	1	2	3
Number			
Weight (g)	125	194	206
Liver			
Weight (g)	4.10	5.70	6.80
Volume (cm^3)	3.83	5.33	6.35
Density (g/cm^3)*	1.070	1.069	1.071
Reference volumes (% liver volume)			
Parenchyma	92.37	92.37	92.37
Hepatocyte cytoplasm	72.55	69.28	72.05

* Determined after Scherle (28).

STAGE II: ELECTRON MICROSCOPY, FINAL MAGNIFICATION $\times 6,200$; REFERENCE VOLUME, LIVER PARENCHYMA: This stage was used to determine the volume densities of the hepatocytic nuclei and cytoplasm in the liver parenchyma. 48 electron micrographs were collected and analyzed with a multipurpose test system 27 \times 27 centimeters, containing 168 test points.

STAGE III: ELECTRON MICROSCOPY, FINAL MAGNIFICATION $\times 19,000$; REFERENCE VOLUME, HEPATOCYTE CYTOPLASM: The third sampling stage was used to determine the plasma membrane surface area of hepatocytes. 48 electron micrographs were again collected. Using a square double lattice test system (36:324), intersections between plasma membrane and all horizontal and vertical lines were counted, as well as coarse points falling on hepatocyte cytoplasm.

STAGE IV: ELECTRON MICROSCOPY, FINAL MAGNIFICATION $\times 96,000$; REFERENCE VOLUME, HEPATOCYTE CYTOPLASM: This highest magnification stage was used to determine the surface densities of all cytoplasmic membranes of hepatocytes. A total of 72 electron micrographs were selected for each animal.

The evaluation of cumulative standard errors has shown that the number of electron micrographs chosen for each sampling stage yielded results with a coefficient

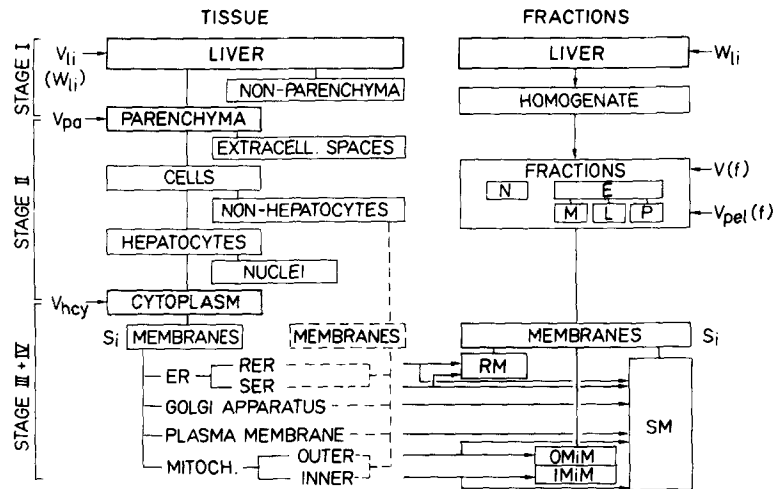


FIGURE 1 Flow chart connecting stepwise the subcellular membrane components of the liver for intact rat liver tissue and fractions. Organelles identified as specific membrane compartments in the intact tissue are identified in the fractions as rough membranes (*RM*) when they carry ribosomes, as smooth membranes (*SM*) when they are fragments without ribosomes, and as mitochondrial membranes (*MiM*) when the inner and outer membranes retain a recognizable configuration. As discussed in the text, all the stereological data are related to the unit liver volume (V_{li}) or weight (W_{li}). The intact tissue reference volumes used to relate hepatocytic membrane surface areas (S_i) measured at Stages III and IV to the 1 cm^3 (or gram) of liver at Stage I included the hepatocyte cytoplasm (V_{hcy}) and the parenchyma (V_{pa}). The pellicle volume ($V_{pel}(f)$) was the volume of a disk produced by filtering a known fraction aliquot ($V(f)$). The fractions included: *E*, extract; *N*, nuclear, *M*, heavy mitochondrial; *L*, light mitochondrial; *P*, microsomal; and *S*, supernatant, after de Duve (11).

of variation of <10% of the mean for all components, except the Golgi membranes.

Tissue Fractions

TISSUE FRACTIONATION PROCEDURE

Six fractions were obtained by differential centrifugation according to Appelmans et al. (1), de Duve (11), and Beaufay (personal communication): E (extract), N (nuclear), M (heavy mitochondrial), L (light mitochondrial), P (microsomal), and S (supernatant). As far as possible, the fractionation procedures duplicate those of the Louvain group.

E AND N FRACTIONS: The liver was minced into a Potter homogenizer (3431-E55; Arthur H. Thomas Co., Philadelphia, Pa.) containing 3 ml of 0.25 M sucrose buffered at pH 7.4, with 3 mM imidazole-HCl per g of liver. It was then homogenized at ~700 rpm for 20 s in one stroke down and up. The homogenate was centrifuged at 4,900 $g \cdot \text{min}$ in an MSE 4L centrifuge (Mistral Crawley, Sussex, England) refrigerated at 4°C. The resuspended N pellet was washed twice ($2 \times 3,800 g \cdot \text{min}$) and adjusted by weight to a dilution 1:5, whereas the E fraction was diluted to 1:10.

M FRACTION: 30 ml of the E fraction was centrifuged at 4°C (31,300 $g \cdot \text{min}$; Beckman L2 65b, rotor 40; Beckman Instruments, Inc., Spinco Div. Palo Alto, Calif.). The resulting pellet was resuspended and washed two times under the same centrifugal conditions, and adjusted to a dilution 1:5.

L FRACTION: The supernate from the preceding step was centrifuged with rotor 40 at 276,200 $g \cdot \text{min}$. The L pellet was resuspended and washed two times under the same conditions and diluted by weight to 1:5.

P + S FRACTIONS: The P + S supernate was centrifuged with rotor at 3,009,840 $g \cdot \text{min}$. After removing the supernate (S), the resuspended pellet (P) was washed two times and diluted by weight to 1:5.

PREPARATION OF FRACTIONS FOR ELECTRON MICROSCOPY

Stereological data were obtained from pellicles produced by collecting known amounts of a fraction on a Millipore filter (Millipore Corp., Bedford, Mass.), as described by Baudhuin et al. (3). For each of the fractions, a 0.1-ml aliquot of a known dilution was mixed with 0.9 ml of an ice-cold fixative containing 1.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4, 340 mosM [adjusted with sucrose]). The suspension was then transferred to a filtration cylinder; 0.5 ml of 0.1 M Na-cacodylate buffer (pH 7.4, 340 mosM [adjusted with sucrose]) was carefully layered over the suspension, and a pellicle was formed in about 20 min by filtering at a pressure of 2.5 atm. The pellicle-Millipore filter-supporting screen "sandwich" was transferred to a threaded plastic holder and covered with a similar Millipore filter. The pellicle was then postfixed in

1% osmium tetroxide buffered with 0.1 M Na-cacodylate (pH 7.4, 340 mosM [adjusted with NaCl]) for 2 h at 0°C and subsequently held overnight in a 0.1 M cacodylate buffer (see above). The following morning, the pellicle was contrasted en bloc with uranyl acetate as described for tissue sample preparation. After ethanol dehydration, the pellicle sandwich was removed from the holder and placed into a small petri dish containing propylene oxide to dissolve the Millipore filter. Finally, the pellicle was embedded flat in Epon disks. The pellicle diameters were measured in both propylene oxide and polymerized Epon and found to be similar. Thin sections were cut perpendicular to the horizontal surface of the pellicle.

STEREOLOGICAL ANALYSIS OF FRACTIONS

The purpose in collecting stereological data from pellicles was to estimate the surface area of membranes in a fraction related to 1 g of liver (Fig. 1). To this end, it was necessary to determine both the volume of the pellicle and the surface of the membranes per unit pellicle volume, and then to relate these measurements to 1 g of liver by considering the amount of fraction filtered.

PELLICLE VOLUME: Pellicle volume was determined from its diameter, D , measured when in propylene oxide-Epon, and its thickness, t , measured on low power electron micrographs of thin sections (Fig. 2).

PELLICLE CONTENT: From thin sections derived from two blocks selected at opposite sides of the pellicle, a row of nonoverlapping micrographs was taken throughout the pellicle thickness as shown in Fig. 2.

Pellicles derived from fractions E, N, M, and L were analyzed at $\times 120,000$ and P at $\times 150,000$, using a square, double lattice test system (1:9), 36 coarse points, 27×27 cm. The combined data associated with one set of micrographs covering the entire pellicle thickness were used as a single sample for statistical analysis. Depending on the pellicle thickness, this set included between 3 and 22 micrographs. For each stage, the size of an individual sample estimate was considered to be adequate when for several consecutive calculations the standard error was <10% of the mean.

Correction of Errors Due to Section Compression

INTACT TISSUE

The compression of sections in the direction perpendicular to the knife edge causes the information contained in the section to be concentrated into a smaller area; as a result, surface densities may be overestimated, whereas volume densities are generally uninfluenced (20). Because the nuclei of hepatocytes are predominantly spherical, the distortion of their profiles to ellipses can be used to estimate the degree of section compression. If a and b are the major and minor axes of elliptic

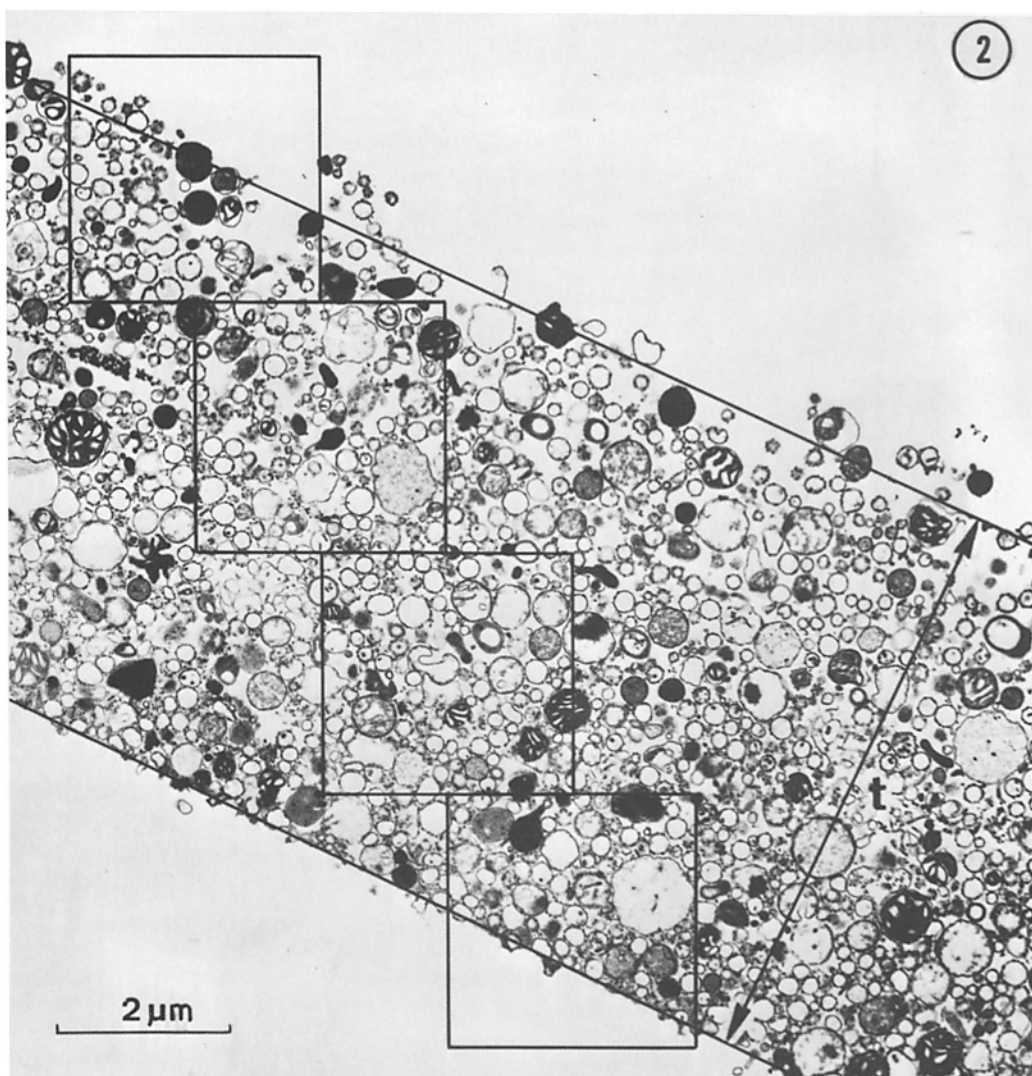


FIGURE 2 Pellicle sampling procedure. Adjacent, nonoverlapping micrographs (squares) were collected covering the pellicle thickness (t). The Millipore filter—no longer present—had been adjacent to the lower surface. $\times 11,500$.

nuclear profiles (Fig. 3) measured perpendicularly and parallel to the direction of sectioning, a correction factor for section compression effects on surface density (S_V) estimates is found, according to Loud (22), by:

$$K_c(S_V) = \frac{(b/a)}{\sqrt{\frac{1 + (b/a)^2}{2}}}$$

The major and minor axes of 300 nuclei were measured from each animal, and the average ratio was used to calculate the correction factor to be 0.905. Disregarding this factor would have yielded overestimates of S_V by

some 10%.

PELLICLES

Stereological data obtained on sections of pellicles are likewise affected by section compression; the surface density of membranes is overestimated. On the other hand, the pellicle or reference volume is underestimated due to the fact that the pellicles were cut with the knife edge parallel to the flat pellicle surface; the pellicle thickness, t , measured on micrographs of these sections, is therefore smaller than the true pellicle thickness. In first approximation, it can be assumed that these two effects compensate each other.

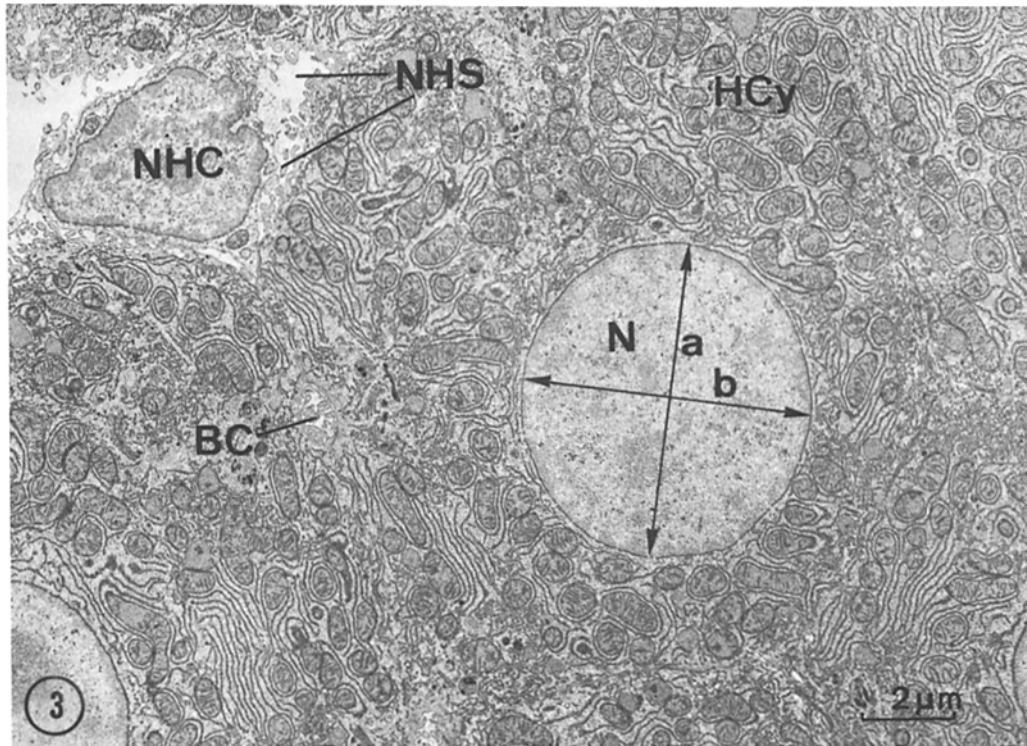


FIGURE 3 A low power electron micrograph of the intact tissue showing hepatocytic nuclei (*N*) and cytoplasm (*HCy*), nonhepatocytic cells (*NHC*) and spaces (*NHS*) and a bile canaliculus (*BC*). Measurements of the major (*a*) and minor (*b*) axes of hepatocytic nuclear profiles were used to estimate section compression. $\times 6,000$.

Correction of Errors due to Section Thickness

The basic stereological formula for estimating S_V is strictly valid only for infinitely thin sections. If it is used on "ultrathin" sections of finite thickness, systematic errors are introduced which depend (*a*) on the shape of the structural objects and (*b*) on their dimension relative to section thickness. Because one and the same cell component may change its shape between intact cells, homogenate, and fractions, a correction of these errors is particularly important in the present study. Thus, ER changes from cisternal and tubular form in the intact hepatocytes (Fig. 4) to predominantly spherical microsomal vesicles in the fractions (Figs. 5-8); similar changes may affect plasma membrane and mitochondria. In the companion paper (34), we have therefore attempted to derive correction factors on the basis of model considerations which would be applicable to a wide range of structures. The models consisted of discrete spherical vesicles, and of nonconvex aggregates of disks and tubules. The formulas derived, their application, and the resulting error estimations are discussed in

the companion paper (34). Table II lists the correction factors used in the present study as well as the parameters required for their calculation. The thickness of the sections was estimated by the fold method of Small (29); the average thickness was 36.7 nm for the tissue sections and 30.2 nm for the pellicles.

Several organelles could not be described by one of the three simple geometric bodies alone, so that compound factors were required (Table II). Measurements revealed that 75% of the plasma membrane surface was present in the form of microvilli, 25% as "flat" sheets; $2/3$ of the Golgi membranes were found as cisternae, and $1/3$ as vesicles or tubules. (It was not important to distinguish between "vesicles" or "tubules" because the correction factors were similar for both structures.) In mitochondria, the estimate of cristae surface was corrected separately from the envelope part of the inner mitochondrial membrane; the correction factor for the latter was the same as for the outer membrane.

In the fractions, smooth and rough membranes occurred both in the form of spherical vesicles and as broad cisternae or large sheets. A compound correction factor for these membranes was calculated for each

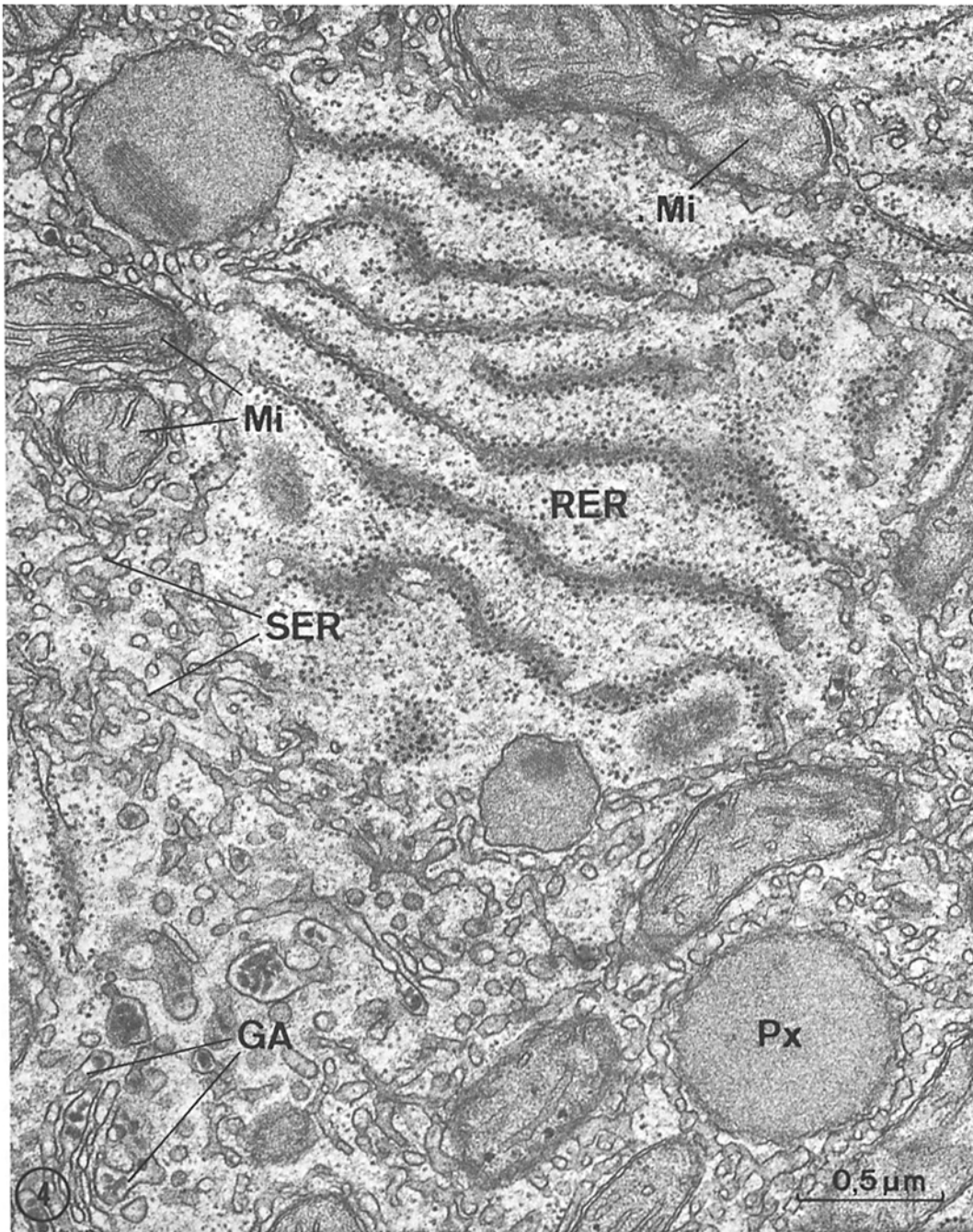


FIGURE 4 An area of hepatocytic cytoplasm illustrating mitochondria (*Mi*), the RER and SER, the Golgi apparatus (*GA*), and peroxisomes (*Px*). $\times 42,000$.

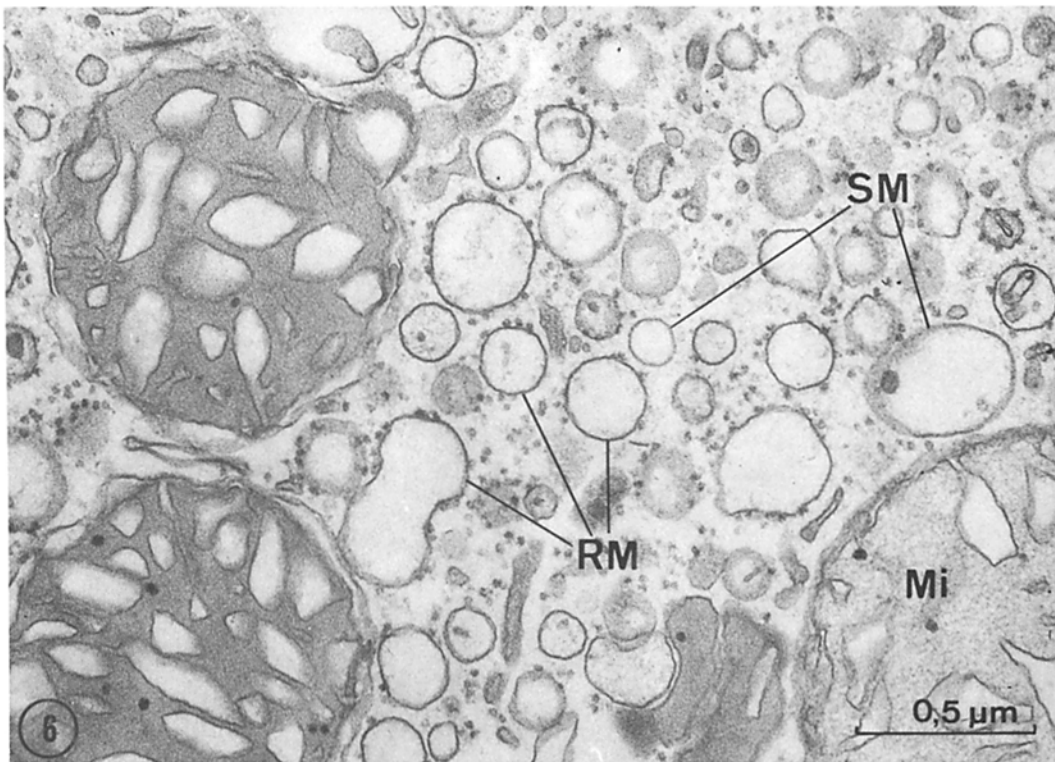
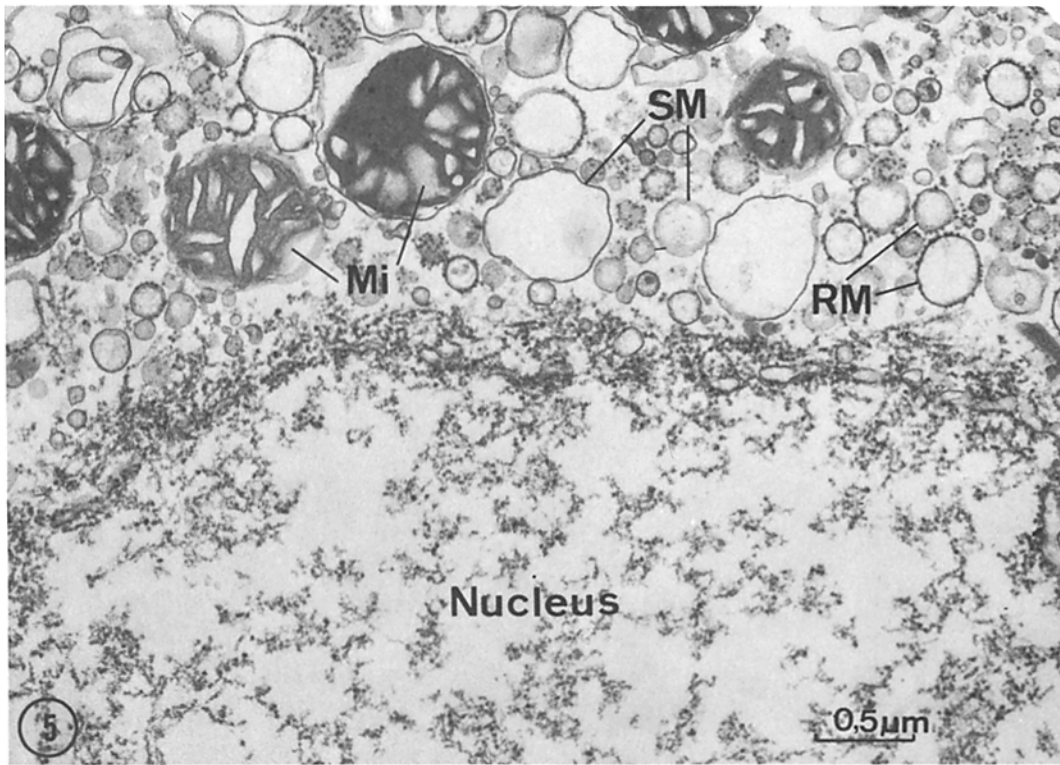


FIGURE 5 N pellicle. The nuclear fraction contains intact and fragmental nuclei, blood cells, mitochondria (*Mi*), and rough- (*RM*) and smooth-surfaced membranes (*SM*) arranged into vesicles. $\times 26,000$.

FIGURE 6 M pellicle. The heavy mitochondrial fraction contains mitochondria (*Mi*) and rough (*RM*) and smooth membranes (*SM*). $\times 48,000$.

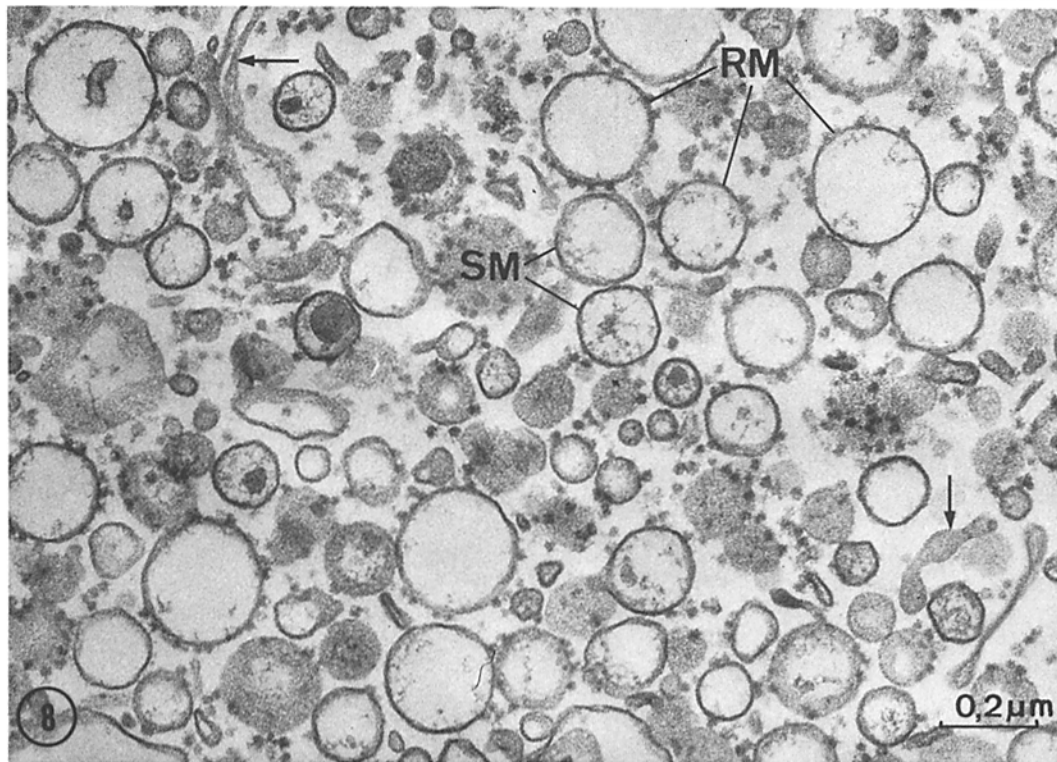
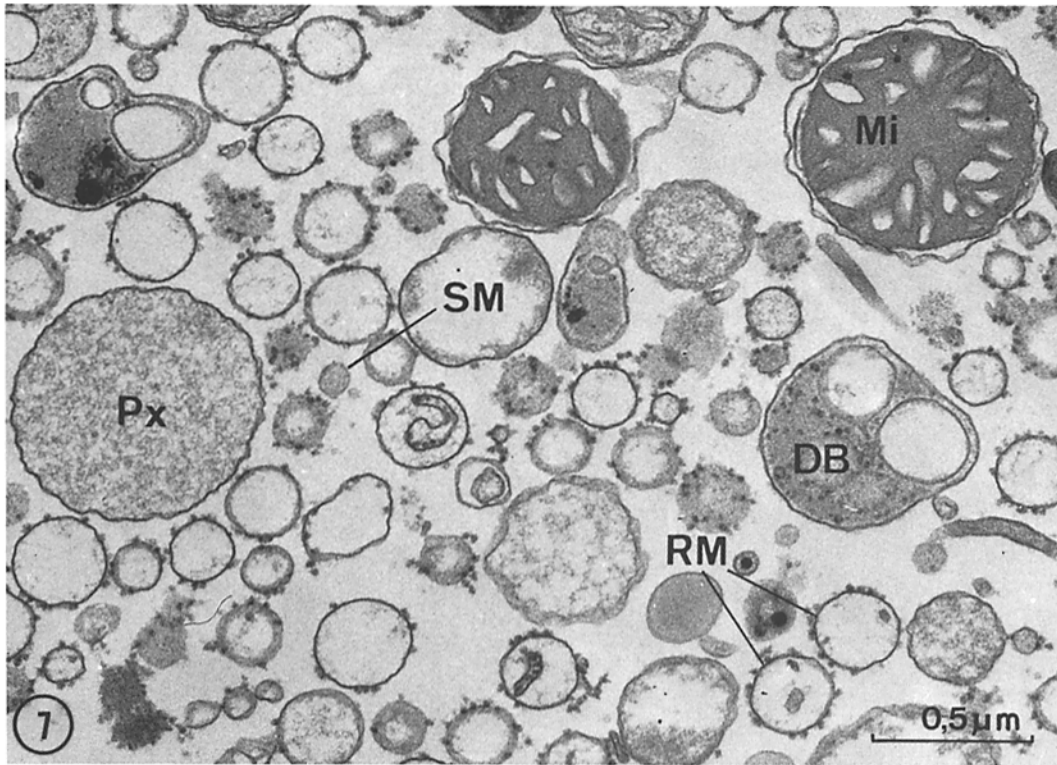


FIGURE 7 L pellicle. The light mitochondrial fraction contains dense bodies (*DB*), peroxisomes (*Px*), mitochondria (*Mi*), and rough (*RM*) and smooth membranes (*SM*). $\times 42,000$.

FIGURE 8 P pellicle. The microsomal fraction contains rough (*RM*) and smooth (*SM*) vesicles as well as irregularly shaped membranes (arrow). $\times 62,000$.

TABLE II
Correction Factors for Section Thickness Effect in Tissue and Fractions

Component	Structural model	Dimensions*			Relative section thickness $g = T/d$	Correction factor $K_c(S)$
					$T = 37 \text{ nm}$	
(a) Intact tissue						
RER	Cisternae	$d = 46$	$D = 2,000$	$\delta = 43$	0.804	0.986
SER	Tubules	$d = 66$	$L = 400$	$\lambda = 6$	0.561	0.702
Golgi apparatus						
Cisternal (67%)	Cisternae	$d = 30$	$D = 1,000$	$\delta = 33$	1.23	0.936
Vesicles (33%)	Vesicles	$d = 50$	$d_0 = 25$	$\rho = 0.5$	0.74	0.531
Total						0.802
Plasma membrane						
Flat part (25%)	Cisternae	$d = 10$	$D = \infty$	$\delta \sim \infty$	4.00	1.00
Microvilli (75%)	Tubules	$d = 100$	$L = 500$	$\lambda = 7$	0.37	0.779
Total						0.834
Mitochondria						
Outer membrane	Vesicles	$d = 800$	$d_0 = 300$	$\rho = 0.375$	0.046	0.966
Cristae	Cisternae	$d = 28$	$D = 200$	$\delta = 6.7$	1.321	0.757
					$T = 30 \text{ nm}$	
(b) Fractions						
Mitochondria						
Outer membrane	Vesicles	$d = 800$	$d_0 = 300$	$\rho = 0.375$	0.038	0.973
Cristae	Cisternae	$d = 65$	$D = 200$	$\delta = 3.1$	0.462	0.824
Rough + smooth membranes						
Cisternae	Cisternae	$d \sim 50$	$D \sim 1,500$	$d \sim 40$	~ 0.60	0.975
Vesicles	Vesicles	$d = 200$	$d_0 = 30$	$\rho = 0.15$	0.150	0.842
(in E, N, P)		$m_2 = 1.078$				
Vesicles (in L, M)		$d = 240$	$d_0 = 30$	$\rho = 0.125$	0.125	0.865
		$m_2 = 1.078$				
Compound factors						
N	50% Vesicles					0.908
	50% Cisternae					
M	50% Vesicles					0.920
	50% Cisternae					
L	90% Vesicles					0.876
	10% Cisternae					
P	100% Vesicles					0.842
E	65% Vesicles					0.888
	35% Cisternae					

* Shape characteristic dimensions are (34): vesicles: d , sphere diameter; cisternae: d , thickness, D , diameter; tubules: d , diameter, L , length. d , D , and L in nm; all other factors are dimensionless.

fraction by estimating the proportion of membrane area present in cisternal and vesicular form, respectively.

Biochemical Methods

Aliquots of the fractions were used to determine the total and specific activities of marker enzymes for ER, plasma membrane, outer and inner mitochondrial membranes.

Glucose-6-phosphatase activity was assayed by the method reported by Hers et al. (16), 5'-nucleotidase by the procedure described by Emmelot et al. (13, 15), monoamino oxidase by the method developed by Baudhuin et al. (2), and cytochrome-oxidase according to the method of Appelmans et al. (1, 10). Proteins

were determined by the method of Lowry et al. (23) using bovine serum albumin as a standard.

RESULTS

Stereological Data on Intact Tissue

The surface densities of the various membrane classes per unit volume of the hepatocyte cytoplasm were converted to membrane area per 1 g of liver by considering the volume density of hepatocyte cytoplasm in whole liver and the specific gravity of liver tissue of 1.07 (Table I). These

TABLE III
Membrane Surface in Intact Tissue*

Compartmental surfaces	Animal	m ² /g Parenchyma			m ² /g Liver	Total membranes mean %
		Uncorrected	Corrected			
			T + C	NHM		
Total membranes (SM + RM + MiM)	1	12.6	9.64	10.26‡	9.48	100
	2	12.4	9.62	10.74‡	9.49	
	3	11.9	9.01	9.69‡	8.95	
Endoplasmic reticulum	1	6.41	4.93	5.27	4.87	49.6
	2	5.56	4.37	4.67	4.31	
	3	6.16	4.68	5.00	4.62	
Smooth-surfaced ER (SM)	1	3.06	1.95	2.06	1.90	19.1
	2	2.31	2.00	1.55	1.44	
	3	3.15	2.00	2.12	1.96	
Rough-surfaced ER (RM)	1	3.35	2.99	3.21	2.97	30.5
	2	3.25	2.90	3.11	2.88	
	3	3.01	2.68	2.88	2.66	
Golgi apparatus (SM)	1	0.191	0.138	0.163	0.151	2.0
	2	0.233	0.169	0.200	0.184	
	3	0.279	0.203	0.239	0.221	
Plasma membrane (SM)	1	0.594	0.448	0.581	0.537	6.5
	2	0.851	0.642	0.833	0.769	
	3	0.556	0.419	0.544	0.502	
Mitochondrial membranes (MiM)	1	5.37	4.12	4.23	3.90	41.9
	2	5.77	4.44	4.55	4.20	
	3	4.88	3.78	3.88	3.58	
Outer membranes (OMiM)	1	1.20	1.05	1.08	0.993	10.9
	2	1.30	1.14	1.17	1.076	
	3	1.17	1.02	1.05	0.969	
Inner membranes (IMiM)	1	4.17	3.07	3.15	2.91	31.0
	2	4.47	3.30	3.38	3.13	
	3	3.71	2.76	2.83	2.61	

* Data are corrected for section thickness (*T*) and compression (*C*). The first two columns consider only hepatocytic membranes, as of the third column a correction for nonhepatocytic membranes (NHM) is introduced.

‡ 0.027 m²/g has been added to account for membranes of pinocytotic vesicles of nonhepatocytic cells (5).

calculations produced the uncorrected data presented in Table III, which were then corrected for section compression and thickness. The contribution of nonhepatocytic membranes to the liver parenchyma was accounted for by multiplying the hepatocytic values by factors derived from the study of Blouin et al. (5), and given in Table IV.

TABLE IV
Correction Factors for Nonhepatocytic Membranes*

RER	1.074
SER	1.058
Golgi apparatus	1.178
Plasma membrane	1.297
Mitochondrial membranes	1.025

* Calculated from data of Blouin et al. (5).

The corrected total membrane surface area in 1 g of liver averaged for the three animals was 9.3 m² (Table III). When subdivided into individual membrane compartments, 49.6% came from the ER, 41.9% from mitochondria, 6.5% from the plasma membrane, and 2% from the Golgi apparatus.

Stereological Data on Fractions

In Table V, the estimates for the total membrane compartment subdivided into the three recognizable membrane types (smooth, rough, and mitochondrial) are given corrected for section thickness; see Table II*b*. Uncorrected, the average overestimate for the total membrane surface area per gram of liver is 0.83 m² in the homogeneous and 1.09 m² in the fractions.

TABLE V
Membrane Surface in Fractions

Compartmental surfaces	Animal	Surface area in fractions*				
		E	N	M	L	P
Total membranes	1	6.91	1.06	2.72	0.663	3.14
	2	6.03	1.19	2.08	0.677	2.93
	3	6.72	1.56	1.82	0.401	4.16
Rough and smooth membranes	1	5.38	0.639	0.927	0.512	3.14
	2	4.25	0.658	0.751	0.597	2.93
	3	5.31	0.871	0.644	0.337	4.12
Smooth membranes	1	2.02	0.306	0.341	0.126	1.11
	2	2.54	0.430	0.337	0.154	1.57
	3	3.21	0.522	0.386	0.104	2.84
Rough membranes	1	3.36	0.333	0.586	0.386	2.03
	2	1.71	0.228	0.414	0.444	1.36
	3	2.10	0.349	0.258	0.233	1.28
Mitochondrial membranes	1	1.53	0.425	1.79	0.151	0.00363
	2	1.79	0.537	1.33	0.0794	0.00511
	3	1.40	0.688	1.18	0.0633	0.03912
Outer mitoch. membranes	1	0.357	0.108	0.394	0.0570	0.00251
	2	0.399	0.124	0.327	0.0240	0.00002
	3	0.352	0.188	0.423	0.0190	0.00860
Inner mitoch. membranes	1	1.18	0.317	1.40	0.0944	0.00011
	2	1.39	0.413	1.00	0.0554	0.00488
	3	1.05	0.500	0.756	0.0442	0.03072

* Data are given in m^2/g liver corrected for 30 nm section thickness by factors given in Table II.

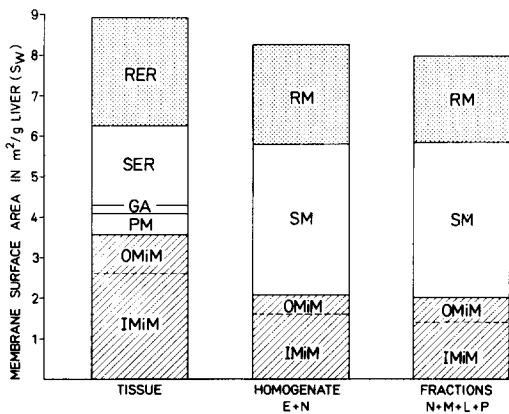


FIGURE 9 The distribution of membranes in the intact tissue, homogenate, and fractions as found in animal no. 3. The total membrane surface area is subdivided into three compartments that can be recognized in all three preparations of the liver: rough (*RM*), smooth (*SM*), and mitochondrial (*MiM*) (*O*, outer, *I*, inner) membranes. In the intact tissue, the *SM* contains membranes from the *SER*, Golgi apparatus (*GA*), and plasma membrane (*PM*), whereas fragments of mitochondrial membranes are added to this compartment in the homogenate and fractions.

To illustrate the distribution of the three recognizable membrane compartments in the fractions and the intact tissue, the data from animal 3 are plotted in Fig. 9. The relative distribution of the rough, smooth, and mitochondrial compartments for the same animal are given in Fig. 10. Although both the mitochondrial and rough-surfaced mem-

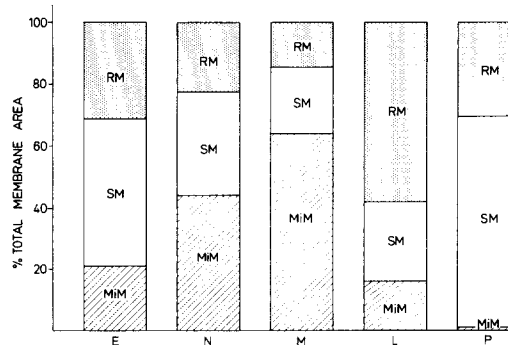


FIGURE 10 The relative distribution of rough (*RM*), smooth (*SM*), and mitochondrial membranes (*MiM*) in the fractions of animal no. 3. The *SM* compartment represented the morphologically unidentifiable membranes in each fraction.

branes of the fractions can be identified with a specific intact tissue compartment, the smooth-surfaced membranes cannot because they contain contributions from the smooth endoplasmic reticulum (SER), Golgi apparatus, plasma membrane, and mitochondrial fragments (Fig. 1). The P fraction contains the largest percentage of nonidentifiable membranes, whereas the M fraction contains the smallest.

Membrane Recoveries: Intact Tissue – Homogenate – Fractions

In Table VI and Fig. 11, morphological recoveries are used to compare estimates for membrane surface areas determined from three preparations of the same liver: intact tissue (T), homogenate (H), and fractions (F). The average values for the total membrane compartments were: (F/H) = 96%, H/T = 84%, and F/T = 81%.

The rough and smooth surfaced membranes exhibited great variations among the animals, but in pooling the two groups (rough plus smooth membranes) these variations disappeared; the recovery of rough and smooth membranes taken together was 100%. In contrast, the mitochondrial mem-

branes consistently showed a loss of over 40% in all three animals, when the fractions were compared to intact tissue; comparing fractions to homogenate, however, the mitochondrial recoveries approached 100%.

Biochemical Data and Recoveries

Table VII presents the total activities of marker enzymes for the predominant membrane classes as estimated in the same preparations as used for the morphological part of this study. The recoveries between fractions and homogenate were 83% for cytochrome oxidase and about 95% for glucose-6-phosphatase, 5'-nucleotidase, monoamine oxidase, and protein. With respect to the distribution of enzyme activities between the fractions, it should be noted that 62% of the glucose-6-phosphatase activity was found in the microsomal fraction, with 7, 12, and 16% in the L, N, and M fractions, respectively. Of mitochondrial marker enzymes, both cytochrome oxidase and monoamine oxidase were concentrated at 68 or 59%, respectively, in the M fraction. It is noteworthy that 14% of the monoamine oxidase activity and 3% of the cytochrome oxidase activity were found in the P fraction.

TABLE VI
Recoveries between Fractions, Homogenate, and Tissue

Compartmental surfaces	Animal	Intact tissue (T)	Homogenate (H)	Fractions (F)	Recoveries (%) mean \pm SE		
					F/H	H/T	F/T
Total membranes	1	9.48	7.98	7.59			
	2	9.49	7.23	6.88	95.5 \pm 0.3	84.3 \pm 4.7	80.5 \pm 4.8
	3	8.95	8.27	7.94			
Rough and smooth membranes	1	5.52	6.02	5.21			
	2	5.26	4.90	4.93			
	3	5.34	6.18	5.97			
Smooth membranes	1	2.59	2.33	1.88	89.3 \pm 7.0	118 \pm 15	107 \pm 21
	2	2.39	2.97	2.49			
	3	2.68	3.73	3.85			
Rough membranes	1	2.97	3.69	3.33	101 \pm 13	94.6 \pm 16.5	92.3 \pm 10.1
	2	2.88	1.94	2.44			
	3	2.66	2.45	2.12			
Mitochondrial membranes	1	3.90	1.96	2.37	99.8 \pm 11.1	55.0 \pm 2.5	54.1 \pm 4.2
	2	4.20	2.32	1.95			
	3	3.58	2.09	1.97			
Outer membranes	1	0.993	0.426	0.526	113 \pm 10	46.3 \pm 2.6	52.2 \pm 5.9
	2	1.076	0.480	0.447			
	3	0.969	0.497	0.601			
Inner membranes	1	2.91	1.49	1.81	96.4 \pm 12.5	56.1 \pm 2.5	53.5 \pm 4.5
	2	3.13	1.80	1.48			
	3	2.61	1.55	1.33			

* Data are given in m²/g liver.

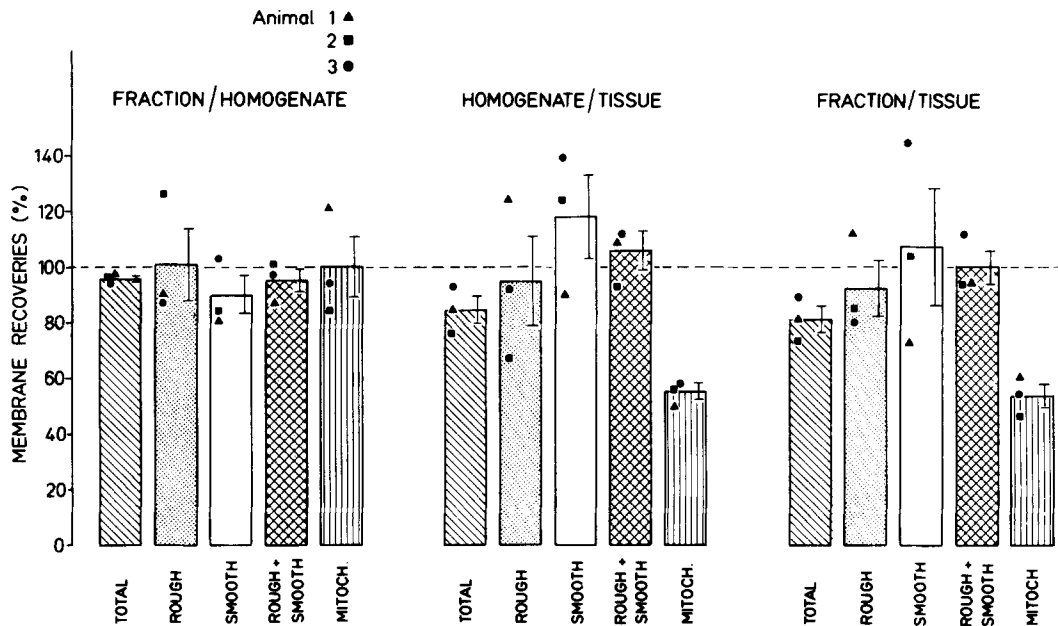


FIGURE 11 Membrane surface area recoveries (100% = perfect recovery) for the three animals, means and standard errors are included. The 96% fraction/homogenate recovery indicates that most of the membrane surface area is conserved during the fractionation. Tissue recoveries of 60% for the mitochondrial membranes indicate the extent to which these membranes are being fragmented and lost to the smooth membrane compartment.

TABLE VII
Biochemical Data from Fractions

Enzymes	E	N	M	L	P	S	Recovery
Glucose-6-phosphatase	22.62 ± 2.802	4.256 ± 0.563	3.639 ± 0.738	2.435 ± 0.2003	14.74 ± 2.734	0.4201 ± 0.0467	94.66 ± 1.21
Cytochrome oxidase	15.53 ± 0.61	4.12 ± 0.46	11.10 ± 0.49	0.70 ± 0.03	0.46 ± 0.06	--	83.47 ± 1.03
Monoamine oxidase	0.506 ± 0.037	0.133 ± 0.029	0.353 ± 0.047	0.020 ± 0.002	0.087 ± 0.009	0.015 ± 0.003	94.30 ± 4.32
5'-nucleotidase	10.75 ± 0.15	5.08 ± 0.72	3.40 ± 0.18	0.85 ± 0.05	4.39 ± 1.14	1.43 ± 0.08	95.11 ± 5.65
Protein	193.21 ± 1.64	48.63 ± 3.32	57.68 ± 2.23	6.96 ± 0.35	39.13 ± 0.93	82.30 ± 0.75	97.05 ± 0.59

Enzymes: U/g liver; proteins mg/g liver. $n = 3 \pm SE$.

DISCUSSION

The purpose of this study was to evaluate morphometric data on liver membranes—collected from intact tissue, homogenate and fractions of the same liver—in the same way that analytical biochemical procedures use recoveries to compare homogenates to fractions (1, 8, 9). Two distinct advantages can be gained by subjecting the morphological data to this type of analysis. First, it opens the possibility to quantitatively correlate morphological and biochemical data, because both are derived from the same homogenate and

fractions, both are related to the same “1 g of liver,” and both are tested for reliability by calculating recoveries. Second, the morphometric analysis allows the membrane content of fractions and homogenate to be linked back to intact tissue, a possibility which does not exist for biochemistry.

Methods

Whereas the basic stereological methods were available from previous work, a number of improvements in the methodology had to be introduced.

CORRECTION FOR SECTION COMPRESSION: Section compression produced about a 10% overestimate for the total membrane surface area in the intact tissue (Table III). However, the fraction data did not require any correction for section compression, because the pellicles had been cut with the knife edge approximately parallel to the filtration direction. Wibo et al. (36) estimated the total surface area of the membranes in a microsomal fraction, using surface densities and the Wicksell transformation (37) as independent methods; their values were corrected for section thickness, but not for compression. Since their pellicles were cut at 45° to the filtration direction, compression may have introduced some systematic errors into both of their surface area estimates.

CORRECTION FOR SECTION THICKNESS: Correction for errors due to section thickness was most important because the configuration of membranes is drastically altered by fragmentation upon homogenization, e.g., ER membranes are present in the form of cisternae and tubules in the intact cells but become predominantly broken up into vesicles of varying size. The errors in stereological estimates due to section thickness depend, however, on the shape of the organelles. This is discussed in detail in the companion paper.

The correction factors derived for intact tissue indicate that rough endoplasmic reticulum (RER) membrane area is overestimated by some 1.4%, mitochondrial cristae by 32%. A considerable overestimation (42%) occurred in the surface density estimate of tubular smooth ER.

Rather large effects of section thickness were observed in the fractions, particularly in the microsomal fraction with its spherical vesicles of varying size. An estimation of vesicle size distribution, however, permitted the calculation of appropriate correction coefficients, which corrected for overestimation in the order of 19% in the P fraction.

This analysis of model systems has shown that some of the previous concepts of section thickness effects have had to be revised. Loud (21) had reasoned that membrane systems such as cisternae of ER or mitochondrial cristae should be underestimated, because the difficulty of recognizing obliquely sectioned elements should lead to a "loss" of up to 1/3 of the membrane; in an earlier study from this laboratory (35), this concept was adopted although the loss was considered somewhat less important. This notion must be revised:

First, considerations on the geometric probability of sectioning have revealed grazing sections of ER cisternae or cristae to be relatively rare events; secondly, the use of en bloc staining with uranyl acetate enhances the contrast so that obliquely sectioned membranes are still recognized to a large extent particularly if the analysis is done at high magnification, as in the present study.

Results

INTACT TISSUE DATA: The surface areas of hepatocytic membranes have been estimated in several stereological studies, but there has been a considerable lack of agreement between the values reported (7, 21, 25, 27, 35). In addition to differences in animals and preparation procedures usually thought to be responsible for these variations, we can now add errors due to section thickness and compression, as well as variations resulting from the use of different magnifications.

One of the largest discrepancies has been associated with estimates for the ER. By use of the uncorrected data from Table III, the results of this study are more directly comparable to those that did not apply corrections for section thickness and compression. The estimate for ER in this study is 6 m²/cm³. This value is considerably lower than earlier estimates from this laboratory (10.6 m²/cm³ [35] and 8.6 m²/cm³ [7]), slightly higher than those of Loud (4.3 m²/cm³ [21]) or Rohr et al. (4.9 m²/cm³ [27]), but almost the same as in a companion study of Blouin et al. (5.5 m²/cm³ [5]) on perfusion-fixed livers. The initial higher estimates were possibly the result of collecting data from thicker, more compressed sections.

A recent study of Keller et al. (19) has revealed that the estimates of surface density may be resolution-dependent, that is, at higher magnification the details of membrane configurations become more apparent which leads to higher estimates for surface densities. This effect can offer a partial explanation for the above-mentioned differences. In this study, membrane areas were measured at about × 100,000, whereas Loud (21) used × 12,500 for ER measurements and × 50,000 for mitochondrial cristae. On the basis of the findings of Keller et al., it can be estimated that Loud's data on ER should be some 30% lower than those of our previous studies, which is about the order of magnitude in the differences observed.² It is our opinion that higher

² In a preliminary study, we have found that the esti-

magnifications are required for resolution of "relevant detail" of membrane configurations as they are found, for example, in the tubular network of SER.

The estimate for the surface density of the ER corrected for systematic errors was 4.6 m² per g of liver, a value that compares favorably with the 3.8 m²/g found by Blouin et al. (5). On the other hand, Wibo et al. (36), using sections corrected for thickness, estimated that 1 g of liver contains 7.5 m² of ER. This value was obtained by estimating the surface area of the membranes in the microsomal fraction and then extrapolating to the homogenate by using the activity of an ER marker enzyme, glucose-6-phosphatase. This estimate for just the ER was about equal to what we found for all membranes in the homogenate (7.3 m²/g, Table VI) and 63% larger than our intact tissue value for the ER. Since the estimate for the surface area of the microsomal membranes is similar for both studies, the large discrepancy may be related to the extrapolation method. One way to explain the larger estimate in Wibo's study would be to suggest that the ER membranes of the microsomal fraction contain a lower concentration of glucose-6-phosphatase activity than those of the earlier fractions; this interpretation is consistent with some yet unpublished data, wherein glucose-6-phosphatase activity of the fractions was related to the corresponding surface area of the ER.

An important consideration was also the fact that nonhepatocytes may contribute a substantial amount of membranes to the homogenate because they contain about 10% of the total parenchymal membranes. In the present study, the morphometric evaluation of membranes in intact tissue was restricted to hepatocytes only; the possible contribution of nonhepatocytic cells to the total parenchymal membrane pool was introduced by means of correction factors derived from a study of the distribution of membranes to the various cell types (5).

FRACTION DATA: In the stereological analysis of homogenate and fractions, only RER membranes and mitochondrial membranes can be unambiguously identified on sections. About 50% of

mated surface density of inner mitochondrial membrane increases from 3.37 to 4.88 m² per cm³ hepatocytic cytoplasm, i.e., by ~40% if the evaluation is done at $\times 50,000$ or $\times 100,000$, respectively (D. Paumgartner and G. Losa, unpublished results).

the hepatocytic membranes can no longer be identified as belonging to specific membrane compartments. Instead, they become grouped together within a heterogeneous smooth-surfaced membrane compartment which includes the SER, Golgi apparatus, plasma membrane, and probably fragments of other organelles such as mitochondria.

Recoveries

The purpose in determining recoveries for membranes of the fractions is the same as for biochemical data (6, 8, 9), namely, to see how the fractions compare to the homogenate. If the activity of a membrane-bound marker enzyme is conserved during the fractionation procedure, then an equivalent amount of activity should be found in the homogenate (E + N), and fractions (N + M + L + P + S). Likewise, if the fractionation procedure does not influence the surface area of membranes, then a similar amount of membranes would be expected in both the homogenate (E + N) and membrane-containing fractions (N + M + L + P). The 96% recovery of membranes between fractions and homogenate (F/H; Table VI) indicates that this, in fact, was the case. This membrane recovery compares most favorably with published marker enzyme data (1, 6), as well as with those of the biochemical portion of this study (Table VII).

When, on the other hand, the surface area of membranes in subcellular fractions or in homogenate is compared with that in the intact tissue, it becomes evident that the membranes in fractions are not fully representative of those in the intact tissue. It appears that 81% of the total membranes is recovered in the fractions; but there is, by contrast, a striking loss of mitochondrial membranes, the recovery being only 55%, whereas smooth-surfaced membranes appear increased by about the same amount, their recovery being 107%. This latter group primarily comprises membranes derived from SER, Golgi apparatus, and plasma membrane which can no longer be unambiguously identified once the tissue is homogenized; it may, however, also receive substantial contributions from other organelles, particularly those damaged during homogenization.

Considering that the recovery of mitochondrial membranes from homogenate to fractions is 100%, on the average, the loss of mitochondrial membranes and the increase in smooth membranes seem to be related to fragmentation of

mitochondria upon homogenization. In intact hepatocytes, the majority of mitochondria are long "threadlike" bodies that may be of quite complex shape; in fractions, however, they appear to be mostly spherical. This can be explained either by swelling or by fragmentation; the fact that the diameter of mitochondria in fractions is close to the diameter but much smaller than the length of intact mitochondria, makes fragmentation the more likely process. This is further supported by the findings of greater mitochondrial number (as questionable as this parameter may be) and smaller mean volume in fractions (4, 35). Fragmentation could, however, split off variable parts of mitochondrial membranes which would then form vesicles or sheets, morphologically no longer identifiable as of mitochondrial origin; in our analysis, they would be assigned to the class of "smooth membranes." In this respect, it is noteworthy that about 14% of the marker enzyme for outer mitochondrial membrane, monoamine oxidase, is recovered in the P fraction; the morphometric data show, however, that this fraction is composed essentially of vesicles with very few identifiable profiles of mitochondria as contaminants. Fragmentation of mitochondrial membranes and the ensuing formation of unidentifiable smooth vesicles therefore appears the most likely explanation of the apparent loss of mitochondrial membranes and the commensurate increase in smooth membranes.

An alternative explanation must, however, be considered: Mitochondrial membranes might "retract" after homogenization and fragmentation; while still completely within the mitochondrial compartment, they would cover a smaller surface. It is difficult to completely exclude such a possibility on the evidence available from the present data. It seems, however, less important than the loss of recognizable mitochondrial membranes, for the following reason: If enzyme densities are roughly calculated by dividing the monoamine oxidase or cytochrome oxidase activities by the measured mitochondrial surface areas, we find about equal values for the M and L fractions, but values about 50 times higher for the P fraction. On the basis of the retraction hypothesis, this should be interpreted to mean that the (few) mitochondrial membranes in the P fraction have "compacted" their enzymes 50-fold; this is highly unlikely. This rough calculation cannot exclude altogether the possibility of some mitochondrial membrane retraction; however, it makes the alterna-

tive hypothesis appear more likely, namely that some 30% of the mitochondrial membranes become converted to unidentifiable smooth membranes on homogenization. But this warrants further study.

The recovery data (Table VI) suggest that the surface area of RER relative to total membranes recovered increases as a result of homogenization. Since the RER (rough membrane) was identified by the presence of one or more ribosomes bound to a membrane profile, vesicles derived from the transition between RER and SER would be identified more often as RER. Therefore, we cannot conclude that a real increase in RER surface has occurred.

The loss of 19% of total membranes in subcellular fractions could be caused by (a) destruction or retraction of membranes, (b) loss during the preparation procedure; (c) alternatively, it may be only an apparent loss if membranes in the intact tissue were overestimated due to shrinkage of the intact tissue caused by fixation, dehydration or embedding, as found, for example, by Drochmans et al. (12) for isolated hepatocytes. A shrinkage of the tissue volume after the determination of liver weight would lead to an overestimation of membrane surface area; a shrinkage of pellicles would not influence the fraction data as the pellicle volume is measured after embedding. It is at present not possible to establish conclusively which of these effects are the cause of this apparent membrane loss, but the possible contribution of shrinkage must be taken into serious consideration.

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