

# NUCLEAR MEMBRANES FROM MAMMALIAN LIVER

## I. Isolation Procedure and General Characterization

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### ABSTRACT

Nuclear membranes were isolated from rat and pig liver by sonication of highly purified nuclear fractions and subsequent removal of adhering nucleoproteins in a high salt medium. The fractions were examined in the electron microscope by both negative staining and thin sectioning techniques and were found to consist of nuclear envelope fragments of widely varying sizes. Nuclear pore complex constituents still could frequently be recognized. The chemical composition of the nuclear membrane fractions was determined and compared with those of microsomal fractions prepared in parallel. For total nuclei as well as for nuclear membranes and microsomes, various enzyme activities were studied. The results indicate that a similarity exists between both fractions of cytomembranes, nuclear envelope, and endoplasmic reticulum, with respect to their RNA:protein ratio and their content of polar and nonpolar lipids. Both membranous fractions had many proteins in common including some membrane-bound enzymes. Activities in Mg-ATPase and the two examined cytochrome reductases were of the same order of magnitude. The content of cytochrome *b<sub>5</sub>* as well as of P-450 was markedly lower in the nuclear membranes. The nuclear membranes were found to have a higher buoyant density and to be richer in protein. The glucose-6-phosphatase and Na-K-ATPase activities in the nuclear membrane fraction were very low. In the gel electrophoresis, in addition to many common protein bands, some characteristic ones for either microsomal or nuclear membranous material were detected. Significant small amounts of DNA and RNA were found to remain closely associated with the nuclear envelope fragments. Our findings indicate that nuclear and endoplasmic reticulum membranes which are known to be in morphological continuity have, besides a far-reaching similarity, some characteristic differences.

### INTRODUCTION

Many membrane systems of the mammalian liver, including plasma membranes (reviewed in reference 6), the endoplasmic reticulum (reviewed in references 63, 73, 89), and the membranes of the

Golgi apparatus (e.g. 25, 61, 62), have been intensively investigated in the past decade. However, for a variety of technical reasons the nuclear envelope has received relatively little attention. Nu-

clear envelope fragments were isolated from mammalian liver first by the method of Franke (26, 27) which uses a combination of hypotonic shock and sonication for the nuclear disruption (15, 26–29, 95) and, recently, by other authors employing similar methods (9, 43, 97). Other attempts for obtaining nuclear membrane material from diverse mammalian cells, such as HeLa cells, calf thymus, and rat liver, involved the use of detergents such as deoxycholate and Triton X-100 (4, 75, 92, 93) which obviously can cause considerable membrane dissociation and thus progressive loss of membrane material, especially in the outer nuclear membrane (40), and consequently have a very limited range of application.

The present study describes a different method for isolating larger quantities of nuclear envelope fragments from mammalian liver in which the removal of nucleoproteins is achieved by incubating the sonicated nuclear fraction in a high salt medium (cf. also reference 59). For comparison, microsomal fractions were prepared and examined in each experiment by using the same treatments.

The chief aims of the present study were: first, to compare the properties of two different cytoplasmic membranes of the same tissue; secondly, to examine the species variability for two mammals, rat and pig; and thirdly, to make a comparative review of our data and the partially very divergent ones of other authors.

## MATERIALS AND METHODS

### *Isolation Procedures*

**NUCLEI:** Adult pigs and albino Wistar rats (both sexes) which had been fed ad libitum were used. Livers were removed immediately after sacrifice of the animals, cut into slices, and incubated in isolation medium A (0.4 M sucrose; 0.07 M KCl; 2% gum arabic; 0.004 M *n*-octanol; 0.01 M Tris-acetate buffer, adjusted to pH 7.2). The gum arabic was freshly purified by a methanol-modification of Kuehl's procedure (45). When necessary, the liver tissue was freed from connective tissue by careful scraping. All these and the following procedures were carried out in the cold (0°–5°C). Incubation times varied from 2 to 8 hr. The tissue material was homogenized with a rotating knife homogenizer (Fa. E. Bühler, Tübingen, Germany) by four runs at 40,000 rpm for 2–3 sec each. The homogenate was centrifuged at 1500 *g* for 20 min in the 250 or 500 ml centrifuge bottles of the WKF G50K cooling centrifuge (Fa. Weinkauf, Brandau, Germany). The supernatant was collected and used for preparing microsomes as described

later. The pellet containing the nuclei and a certain amount of contaminating whole cells was resuspended in medium B (1.0 M sucrose; 0.07 M KCl; 1% gum arabic; 0.004 M *n*-octanol; 0.01 M Tris buffer, pH 7.2), homogenized with 3–5 strokes in a Potter-Elvehjem homogenizer, and centrifuged again at 2000 *g* for 30 min. The pellet obtained was mixed with the 1.4-fold volume of medium C (3.0 M sucrose; 0.07 M KCl; 0.01 M Tris-acetate buffer, pH 7.2) and spun at 110,000 *g* for 40 min in a WKF P50K ultracentrifuge. The pellet was resuspended in medium A and centrifuged for 15 min at 2000 *g*. The pellet retrieved in this step was resuspended in a small amount of medium A, then mixed thoroughly with four times the volume of medium D (same as C, but 2.2 M with respect to sucrose) and subsequently layered over an equal amount of the nondiluted medium D. This two-layer system was spun at 100,000 *g* for 90 min. The pellet representing a highly pure nuclear fraction was collected, diluted with at least a 10-fold volume of medium A, and finally sedimented at 1500 *g* for 20 min. The purity of this nuclear fraction was routinely monitored in the phase-contrast microscope, and the contamination index, i.e. the total percentage of visible nonnuclear particles, never exceeded 5% (38). The purity and structural integrity of the nuclei were routinely checked with the electron microscope (38). Preparation of the fractions for observation by electron microscopy was performed according to the procedures previously described (98). The number of nuclei used for each biochemical assay was determined by counting nuclei in aliquots in a hemocytometer. The total yield of nuclei in the isolation described was approximately  $3.5 \times 10^8$  nuclei per g fresh weight of liver tissue for pig liver and  $2 \times 10^8$  for rat liver (38).

**NUCLEAR MEMBRANES:** Nuclei obtained from the above described procedure were disintegrated by suspension in 0.3 M sucrose solution (medium E), which was 0.135 M with respect to KCl and 0.01 M with respect to McIlvaine's citrate-phosphate buffer, pH 7.4, and by sonication (10 times at position 5 of the Branson Sonifier S 125 (Branson Instruments Co., Stamford, Conn.) for 2 sec each with 15 sec cooling intervals). To this suspension, a 20-fold volume of the high salt "extraction medium" (0.3 M sucrose; 1.5 M KCl; buffered as medium E) was immediately added, and the suspension was kept in a cold room at 4°C for 5–6 hr during stirring. Then the suspension was centrifuged for 20 min at 1000 *g* to remove intact nuclei, nucleolar fragments, etc. Supernatants from several runs were pooled and spun at 110,000 *g* for 1 hr. The supernatant was discarded and the pellet was resuspended in a small amount of medium E with brief sonication and layered on the top of a linear, continuous 30–70% (w/v) sucrose gradient which was 0.07 M with respect to

KCl and 0.01 M with respect to McIlvaine's buffer (pH 7.4). After 5–6 hr of centrifugation at 80,000 *g* the nuclear membrane material banded sharply and was collected by either dropping or sucking according to the conventional methods.

**MICROSOMES:** Microsomal fractions (i) were prepared from the supernatant of the first centrifugation step of the nuclear isolation described above, or (ii) were prepared by the method of Dallner et al. (20) with slight modifications. (i). The supernatant was centrifuged twice for 20 min in medium A at 8000 *g*. The postmitochondrial supernatants thus obtained were centrifuged again at 110,000 *g* for 90 min. The microsomal pellet was resuspended in 0.85 M sucrose, which was 0.01 M Tris-acetate buffered at pH 7.4 and 0.07 M with respect to KCl, and centrifuged for 2 hr at 110,000 *g* to obtain the final microsomal pellet. (ii) Liver pieces were incubated in 0.4 M sucrose and immediately homogenized with the rotating knife device (5 sec at 40,000 rpm). The filtered homogenate was centrifuged twice at 8000 *g* for 20 min. The postmitochondrial supernatant was adjusted with 1.6 M sucrose to a final sucrose concentration of 0.85 M and centrifuged for 2 hr at 110,000 *g*. The supernatant was discarded and the pellet was resuspended under repeated agitation in 0.4 M sucrose. This suspension was layered on top of a 0.85 M sucrose solution and centrifuged in swinging buckets for 3 hr at 80,000 *g*. The supernatant was decanted, and the pellet was sucked off from the bottom of the tube under resuspension by using a small volume of 0.4 M sucrose solution. This microsomal fraction was finally pelleted for 1 hr at 110,000 *g*.

The microsomes obtained after (i) and (ii) were then resuspended in high salt extraction medium and further treated in the very same way as described above for the nuclear membranes.

### *Electron Microscopy*

The fractions obtained were fixed with 0.05 M cacodylate-buffered (pH 7.0) 1% glutaraldehyde and 1% OsO<sub>4</sub>—either sequentially or simultaneously as described elsewhere (33)—and were postosmicated with 2% OsO<sub>4</sub> for 3–4 hr, dehydrated through graded ethanol solutions, and embedded in Araldite. Some samples were also fixed only with cacodylate-buffered (pH 7.0) 1% OsO<sub>4</sub>. Thin sections were cut on a Reichert ultramicrotome OmU 2 (American Optical Corp., Buffalo, N. Y.) and double stained with uranyl acetate and lead citrate.

Microsomal and nuclear membrane fractions were also routinely examined by using the negative staining technique (1 or 2% phosphotungstic acid, adjusted to pH 7.0) either after 15 min stabilization with OsO<sub>4</sub> followed by several water-washes or directly without any prefixation. Micrographs were taken with a Siemens Elmiskop IA.

### *Chemical Determinations*

After extracting the lipids according to the methods described in a following paper (44), proteins and nucleic acids were precipitated with 10% cold trichloroacetic acid (TCA) and washed four times with the acid. The precipitate then was extracted with hot (90°C) TCA with one wash, and in the extract RNA and DNA were determined by using the orcinol (58) or the diphenylamine (10) reaction. In the residue of the TCA extraction, protein was determined by means of the nitrogen determination by Strauch (87). In the following, hot TCA-insoluble protein is referred to as total protein. Total phospholipids were determined by the lipid-phosphate method.

### *Enzyme Assays*

**ATPase ACTIVITY:** This was determined by measuring the released inorganic phosphate according to Lowry and Lopez (49). The reaction was started by addition of Tris-buffered ATP (5 mM) to the reaction tube containing 5 mM MgCl<sub>2</sub>, 100 mM NaCl, and 10 mM KCl, 20 mM Tris buffer (pH 7.5), and membrane suspension (less than 0.2 mg protein/ml) in a total volume of 1.5 ml. The reaction was carried out at 37°C for 30 min. For measuring the Mg<sup>++</sup>-ATPase activity alone, the reaction mixture additionally contained 10<sup>-4</sup> M ouabain. Control experiments were made without NaCl and KCl, without enzyme, and without substrate.

**GLUCOSE-6-PHOSPHATASE ACTIVITY:** This was measured with glucose-6-phosphate (20 mM) as substrate in 20 mM sodium cacodylate buffer, pH 6.0, at 37°C. Incubation time was 15 min. The inorganic phosphate was determined as described for the ATPases. Some assays were carried out in the presence of 10 mM mercaptoethanol, 5 mM MgCl<sub>2</sub>, using 50 mM Tris-HCl buffer (pH 6.6).

**ALKALINE AND ACID PHOSPHATASE ACTIVITIES:** These were measured according to Linhardt and Walter (48), with *p*-nitrophenyl phosphate as substrate.

**GLUTAMATE DEHYDROGENASE:** The oxidation of NADH in the presence of 2-ketoglutarate, NH<sub>4</sub><sup>+</sup> and EDTA was measured at a wave length of 365 nm according to Schmidt (78).

**NADH- AND NADPH-CYTOCHROME REDUCTASE:** The activities of these were determined as described by Mahler (53). The enzyme contents in the aliquots were calculated assuming a molar extinction increment of 27.7 mm<sup>-1</sup> cm<sup>-1</sup> (70) for reduced cytochrome at 550 nm.

**CYTOCHROME B<sub>5</sub> AND CYTOCHROME P-450:** The content of these in the membranes was measured with a Cary 14 spectrophotometer (Cary Instruments, Monrovia, Calif.) as described by Strittmat-

ter and Umberger (79). The molar extinction increments for these two pigments were taken from Omura and Sato (65). Protein was measured by the method of Lowry et al. (50), with crystalline bovine plasma albumin as standard.

### *Disc Electrophoresis*

The membranes were dialyzed for 20 hr against distilled water containing 6 mM mercaptoethanol which had been brought to pH 9.0 with  $\text{NH}_4\text{OH}$  as modified from the procedure of Mazia and Ruby (57). After 20 hr of dialysis the membranes were centrifuged for 30 min at 100,000 *g*. Supernatant and pellet were used for electrophoresis at basic and acidic pH, respectively. The pellet was solubilized in phenol:urea:acetic, 2:1.2:1 (w/w/v) following the prescription of Takayama et al. (88).

Disc electrophoresis of the supernatant was carried out on the polyacrylamide gel system No. 1 of Maurer (56), with the following modifications: all buffers and the lower gel contained 6 M urea and the agents Na-EDTA (1.25 mM), Na-dodecylsulphate (0.1%), Triton X-100 (0.1%), and mercaptoethanol (5 mM), and had been brought to pH 9.0. No upper gel was used.

Gel electrophoresis of the pellet material was carried out on the system described by Takayama et al. (88). Gels were stained with Amido Black. Application of the sedimentable material to the Maurer No. 1 system did not yield recognizable banding. On the other hand, the Takayama system was unsuccessful with the supernatant material.

## RESULTS

### *Nuclear Fractions*

The mean RNA, DNA, and protein ratios of the nuclear fractions used in this study were 0.20 (rat) and 0.22 (pig) for RNA/DNA, 0.089 (rat) and 0.087 (pig) for RNA/protein, and 0.42 (rat) and 0.38 (pig) for DNA/protein. These data for

the rat liver nuclei are in fair agreement with those communicated by various other authors using modifications of the Hogeboom-Chauveau method (13; review in reference 74; cf. also references 2, 98). The RNA:DNA ratio, however, is markedly higher than that reported for nuclei obtained after the Blobel—Potter procedure (8). The purity of the fractions and the influence of the single constituents of the isolation media used were previously described elsewhere (38). Since it could be repeatedly established that addition of bivalent cations to our basal medium did not result in any significant changes in structure or chemical composition (38), such ions were omitted in most experiments.

Particular care was taken to maintain optimal structural integrity of the isolated nuclei, especially with respect to the preservation of the nuclear envelope as judged by electron microscopy (cf. also reference 32). The preservation of the nuclear envelope is documented in Figs. 1–5. Not only are both the inner and outer nuclear membranes well preserved over almost the entire nuclear surface (Fig. 1), but also all ultrastructural components of the pore complex (28, 30) can still be recognized including the inner pore material (see also reference 52). The central granule material (Figs. 3 and 5), the annular subgranules (Fig. 3), and the inner pore material (Fig. 4) are usually seen. It is noteworthy that the outer nuclear membrane after this type of isolation is still densely occupied by ribosomes (Fig. 2).

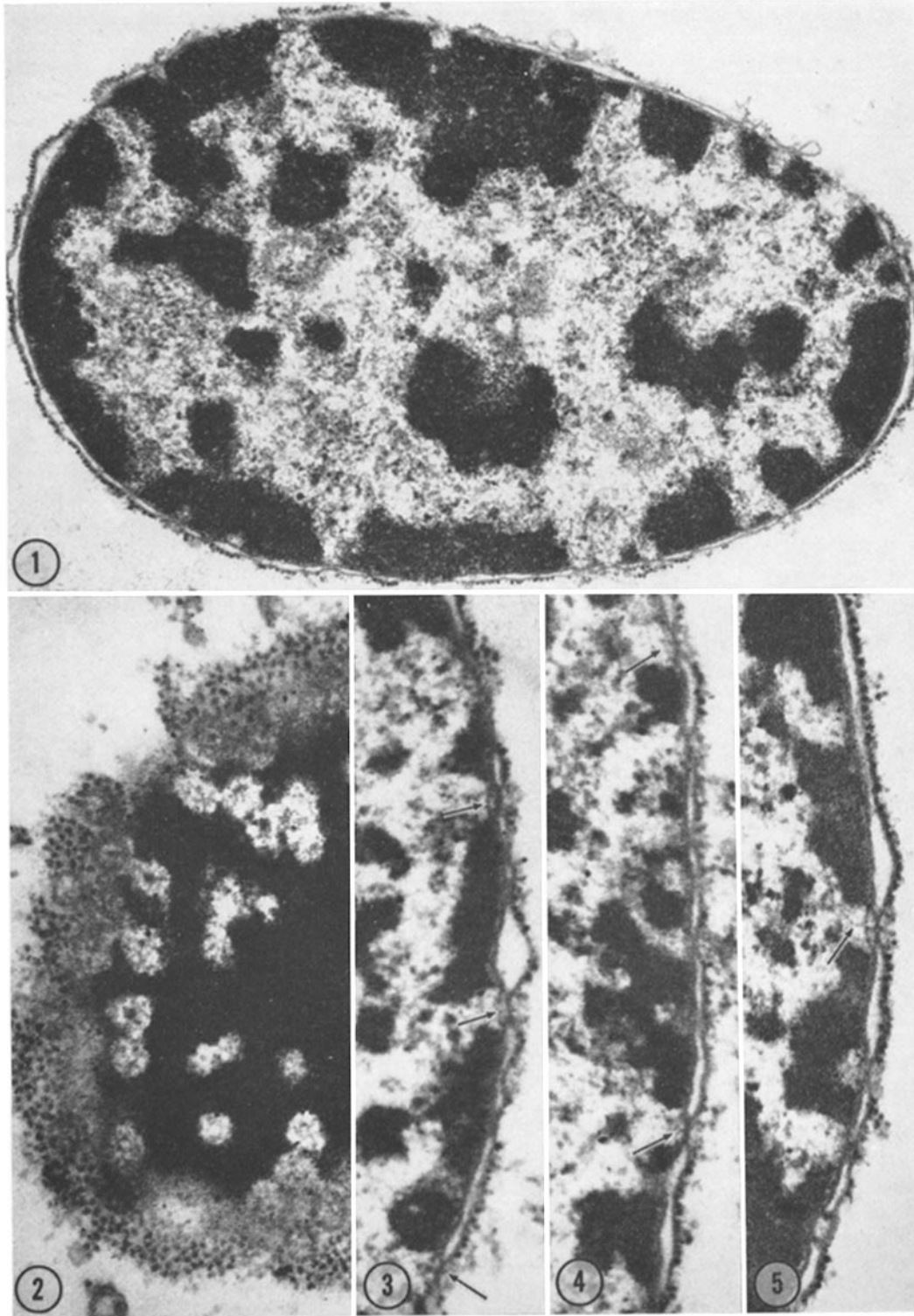
### *Density of Microsomes and*

### *Nuclear Membranes*

In the 24–70% sucrose gradient the nuclear membranes band rather sharply in a single band and sediment significantly deeper than the mi-

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FIGURES 1–5 Structural preservation of the nuclear envelope after the nuclear isolation procedure described in the text. A typical whole nucleus thus isolated from pig liver is shown in Fig. 1, exhibiting “normal” internal nuclear structures as well as an almost entirely intact envelope. In sections tangential to such isolated nuclei the outer membrane of the nuclear envelope is seen as crowded by ribosomes (Fig. 2). Cross-sections through the peripheral chromatin reveal dense particulate material in the center of the chromatin channels interpreted as cross-sections through the central rods or central fibrils which are in continuity with the central granule of the pore complex. Preservation of the nuclear envelope is demonstrated in more detail in Figs. 3–5 in which inner pore material can be recognized as e.g. the annular granules lying on either side at the pore margin (Fig. 3, middle arrow), the dense nonparticulate matter within the pore (Fig. 4, arrows), and the central granules (Fig. 5, arrow; also Fig. 3, lower arrow). The central granule is frequently seen in fibrillar continuity with thin intranuclear filaments (Fig. 5, arrow). Fig. 1,  $\times 34,000$ ; Fig. 2,  $\times 70,000$ ; Figs. 3 and 4,  $\times 60,000$ ; Fig. 5,  $\times 69,000$ .



osomes from the same tissue which occupy a wider range of density. The mean peak densities ( $\rho_4^{22}$ ) of the nuclear membranes after isopycnic banding were determined to be  $1.215 \pm 0.010$  (rat) and  $1.203 \pm 0.016$  (pig). The buoyant densities of the microsomes in our preparations ( $1.183 \pm 0.016$  for rat and  $1.172 \pm 0.014$  for pig material, respectively) correspond to the values reported by other authors for a comparable, relatively "smooth" state of ER membranes (e.g. 20, 63). The marked density difference between microsomal and nuclear membranes was apparent in both pig and rat liver. Splitting of the nuclear membrane material into a lighter and

a heavier subfraction, as was described for the discontinuous sucrose gradient separations by Zbarsky et al. (97) and Kashnig and Kasper (43), was only occasionally observed with our rat liver preparations. One finding of general interest for the technique of membrane separation and characterization in sucrose gradients was the observation that membranes of different densities can behave in the gradient as true "density hybrids." Thus, e.g., under certain conditions, a low degree of microsomal or plasma membrane contamination may bring about a density shift of the nuclear membrane material to lower values, which indicates that the membranous sheets or vesicles do not generally sediment as single particles but rather as randomly aggregated clusters.

TABLE I  
Gross Composition of Nuclear Membranes and  
Microsomes from Rat and Pig Liver

	Nuclear mem- branes from rat	Nuclear mem- branes from pig	Micro- somes from rat	Micro- somes from pig
	% of Weight			
Protein	75.5	74.8	68.5	63.6
Phospholipids	16.1	18.2	24.6	29.6
Nonpolar lipids	2.8	3.0	4.3	4.9
RNA	3.6	2.8	2.5	1.8
DNA	2.0	1.2	<0.1	<0.1

#### Chemical Composition of the Membranous Fractions

The gross composition of the microsomal and nuclear membrane fractions is given in Table I. For all further comparisons between microsomes and nuclear membranes of this study, it should be kept in mind that the duration of the isolation procedure as well as the media used were almost identical for both microsomes and nuclear membranes. For that reason, a direct comparison of these two membrane fractions can be made.

TABLE II  
RNA, Phospholipid, and Protein Ratios (*w/w*) of Nuclear Membranous and Microsomal  
Fractions from Rat and Pig Livers in Comparison with Some Recent Reference  
Values

	RNA/protein	Phospholipids/protein	Phospho- lipids/ protein (corrected †)
Pig liver, nuclear membranes*	0.036 ± 0.008	0.25 ± 0.02	0.26
Pig liver, microsomes*	0.025 ± 0.007	0.46 ± 0.03	0.47
Rat liver, nuclear membranes*	0.048 ± 0.009	0.22 ± 0.03	0.24
Rat liver, microsomes*	0.040 ± 0.011	0.37 ± 0.03	0.39
Rat liver, whole microsomes (66)	0.23	0.28	0.36
Rat liver, whole microsomes (67)	0.23	0.28	0.35
Rat liver, whole microsomes (20)	0.26	0.23	0.31
Rat liver, rough microsomes (18)	0.18	0.41	0.50
Rat liver, whole microsomes (89)	0.195	0.39	0.48
Rat liver, smooth microsomes (89)	0.013	0.46	0.47
Rat liver, Golgi membranes (62)	0.009	0.39	0.39

\* This study; means from 10 determinations.

† After subtraction of nucleoprotein contamination using a maximal RNA:protein weight ratio of 1.0 as for eukaryotic ribosomal nucleoproteins (84) and a maximal DNA:protein weight ratio of 1.67 as for rat liver chromatin (54).

Table I shows that the nuclear membranes from both pig and rat liver are relatively richer in protein, and thus have a considerably lower phospholipid:protein ratio than microsomes (Table II). The content of nucleic acids which might be regarded as a monitoring value for contamination with adhering nucleoproteins is low. The RNA:protein ratios of all the membrane fractions are lower than 0.05 (Table II) and thus show that the majority of the ribosomal ribonucleoprotein (RNP) has been extracted in the high salt medium. Consequently one is dealing with an "artificially smooth" type of cytomembrane. Attempts to reduce further the nucleic acid content were not successful. This might be explained by an intimately tight association of a special sort of RNA not only to microsomes, the so-called "membrane RNA" (reviewed in references 63, 80), but also to the outer nuclear membrane (and possibly to the inner one, too). A certain part of the nuclear deoxyribonucleoprotein (DNP) seems to be also in a close association with the inner nuclear membrane (Table II). The amounts of nonpolar lipids are comparable in all the membranous fractions examined. With the microsomes, no differences were observed between fractions isolated from the nuclear supernatant (i.e. preparation [i] of Materials and Methods) or after the modified Dallner et al. method (preparation [ii]).

The phospholipid and nonpolar lipid pattern of the membrane fractions under study will be reported in detail in a separate article (44). The cholesterol contents of our fractions were consistent with comparable data for microsomes in the literature (for detailed discussion see reference 44) but differed from the higher value reported by Kashnig and Kasper (43) whose cholesterol:phospholipid ratios would be characteristic for plasma membranes from liver (e.g. 6, 14) rather than for cytomembranous fractions (cf. references 18, 20, 44, 69).

### *Enzyme Activities*

The results of the enzyme tests performed on the three fractions, intact nuclei, nuclear membranes, and microsomes, are listed in Table III. Mg-stimulated adenosine triphosphatase (ATPase) activity was found in all the fractions examined and was especially enriched in the two membranous fractions. However, while a small amount

of Na-K-stimulated ATPase activity was present in the microsomal fractions (cf., however, reference 23), as can be seen from the ouabain inhibition and the increase in the additional presence of the monovalent cations (Table IV), this type of ATPase was almost totally absent in the nuclear membranes. In order to examine any influence of the storage of the membrane fractions on the enzyme activities, we measured the loss of enzyme activity under the storage conditions used ( $-20^{\circ}\text{C}$ ). For example, storage for 7 days resulted in only an 8% loss of enzymatic activity of Mg-ATPase, and determinations for other enzyme activities showed comparable results. Since all enzyme activities in this study were routinely measured within 1 day, any nonspecific loss by aging was considered to be negligible (cf., e.g., also references 1 and 18). The observed lack of Na-K-stimulated ATPase in the nuclear membrane agrees with the results of Zbarsky and his coworkers (22, 97).

Glucose-6-phosphatase, which is generally considered to be a marker enzyme for endoplasmic reticulum membranes, was found in our microsomal preparations in amounts comparable to those reported in the literature (19, 21, 24, 43, 67, 97; for bovine liver cf. also references 25 and 51). The nuclear membranes, however, showed less than 1.5% of the microsomal glucose-6-phosphatase activity in both rat and pig liver. Since, with respect to the results on the occurrence of glucose-6-phosphatase in rat liver nuclear membranes, there exists a total contradiction between the Moscow group (97) and Kashnig and Kasper (43), we should perhaps especially point out our attempts toward a characterization of the phosphohydrolase specificity by making use of the inhibitory effect of glucose (e.g., references 11 and 39) and citrate (e.g., reference 64; see, however, reference 5) as well as by examining the temperature effect (85). The apparent inhibition of phosphohydrolyzing activity with glucose-6-phosphate as substrate in all these assays (Table V) may serve as another indication of the relevance of the observed glucose-6-phosphatase activity differences between microsomes and nuclear membranes.

Differences in the activities of alkaline and acid phosphatases between the three fractions studied were much less pronounced. Although there exist earlier remarks on microsomal and nuclear (membrane) acid phosphatase activity (e.g. 12, 35, 36, 81) at present we would tentatively interpret our

TABLE III  
Some Enzyme Activities of Nuclei, Nuclear Membranes, and Microsomes Isolated from Rat and Pig Liver

Enzyme	Microsomes		Nuclear membranes		Nuclei		Nuclear membranes/nuclei		Nuclear membranes/microsomes	
	Pig	Rat	Pig	Rat	Pig	Rat	Pig	Rat	Pig	Rat
Mg-ATPase*	13.4 ± 7.1	7.3 ± 1.5	6.9 ± 1.0	5.6 ± 0.5	3.6 ± 1.3	1.3 ± 0.5	1.9	4.3	0.5	0.8
Na-K-ATPase*	8.6	3.9	0.2	0.3	2.1	0.3	0.1	1	0.02	0.08
Glucose-6-phosphatase*	23.5 ± 1.7	7.3 ± 1.8	0.1	0.1	0.9 ± 0.4	1.9 ± 0.7	0.1	0.05	0.004	0.014
Alkaline phosphatase*	7.1 ± 1.2	9.2 ± 2.1	2.1 ± 0.6	1.7 ± 0.3	3.1 ± 0.4	2.4	1.7	0.7	0.3	0.2
Acid phosphatase*	52.2 ± 10.5	41.1 ± 4.2	10.0 ± 3.6	3.2 ± 1.5	24.3 ± 8.9	11.0	0.4	0.3	0.2	0.08
Glutamate dehydrogenase†	2.4 ± 0.8	—	1.9 ± 0.7	—	1.7	—	1.1	—	0.8	—
NADH-cytochrome c reductase‡	0.15 ± 0.065	0.35 ± 0.19	0.045 ± 0.023	0.10 ± 0.05	—	—	—	—	0.3	0.3
NADPH-cytochrome c reductase§	0.044 ± 0.010	0.049 ± 0.017	0.021 ± 0.008	0.018 ± 0.010	—	—	—	—	0.5	0.4

\*  $\mu\text{M}$   $\text{P}_i$  released/hr per mg protein.

†  $\mu\text{M}$  NAD oxidized/hr per mg protein.

§  $\mu\text{M}$  cytochrome c reduced/min per mg protein.



TABLE IV  
Activities of Adenosine Triphosphatases in Nuclei, Nuclear Membranes, and Microsomes from Rat and Pig Liver

	Assay without Na <sup>+</sup> and K <sup>+</sup> (Mg-ATPase)	Assay with all three ions (Mg-Na-K-ATPase)	Assay with all three ions plus ouabain (Mg-ATPase)	Na-I-	Ouabain
				stimulated ATPase increase (Na-K-ATPase)	sensitive ATPase (Na-K-ATPase)
<i>μM P<sub>i</sub> released/hr per mg protein</i>					
Rat, microsomes (6)*	7.3 ± 1.5	11.2 ± 1.2	8.2 ± 0.5	3.9	3.0
Rat, nuclei (7)*	1.3 ± 0.5	1.6 ± 0.3	1.4 ± 0.3	0.3	0.2
Rat, nuclear membranes (4)*	5.6 ± 0.5	5.9 ± 0.5	5.5 ± 0.6	0.3	0.4
Pig, microsomes (16)*	13.4 ± 7.1	22.0 ± 6.5	15.2 ± 4.2	8.6	6.8
Pig, nuclei (15)*	3.6 ± 1.3	5.7 ± 1.9	4.0 ± 1.8	2.1	1.7
Pig, nuclear membranes (11)*	6.9 ± 1.0	7.1 ± 0.5	7.1 ± 0.6	0.2	—

\* Parentheses give number of experiments.

TABLE V  
Glucose-6-Phosphatase Activities of Nuclei, Nuclear Membranes, and Microsomes from Pig and Rat Liver

Fraction	Standard assay*	Assay with glucose‡		Assay with citrate§		Standard assay carried out at 0°C	Standard assay incubated without substrate for 1 hr at 30°C before measurement
		activity	% inhibition	activity	% inhibition		
<i>μM P<sub>i</sub> released/hr per mg protein</i>							
Rat, microsomes	7.3 ± 1.8	2.5	66	3.6	49	1.9	0.6
Rat, nuclei	1.9 ± 0.7	6.6	68	0.9	53	0.4	0.5
Rat, nuclear membranes	0.1	0.0	—	0.0	—	0.0	0.0
Pig, microsomes	23.5 ± 1.7	7.4	69	14.2	40	3.0	0.9
Pig, nuclei	0.9 ± 0.4	0.7	22	0.8	11	0.8	0.6
Pig, nuclear membranes	0.1	0.0	—	0.1	—	0.0	0.0

\* Standard assay: 0.02 M sodium cacodylate buffer (pH 6.0), 0.02 M glucose-6-phosphate, 0.08 M sucrose, enzyme suspension. Incubation, 15 min at 37° C.

‡ 0.18 M glucose instead of 0.08 M sucrose.

§ 0.04 M citrate instead of 0.08 M sucrose.

values with this enzyme as an indication of lysosomal contamination. Also, with glutamate dehydrogenase no preferential association with membranous material was observed. Other constitutional components of microsomal membranes, the NADH- and the NADPH-cytochrome *c* reductases, exhibited activities in the nuclear membrane fractions that were about one-third and one-

half, respectively, of those in the corresponding microsomal fractions. However, a comparison of the values for the NADPH cytochrome *c* reductase obtained after our above described type of preparation with some reference data on rat liver shows that such lowered activities in the nuclear membranes are still in the range of values reported by other authors for microsomal material (e.g. 19,

66-68, 72, 89, 97). Similar values for microsomal NADPH-cytochrome *c* reduction in bovine liver have been communicated by MacLennan et al. (51) and, more recently, by Fleischer et al. (25). The contents of cytochrome *b<sub>5</sub>* and cytochrome P-450 were determined for pig liver material at protein concentrations of 1.2 and 1.7 mg per ml suspension and were calculated for the microsomes as 0.25  $\mu\text{M}/\text{mg}$  protein (*b<sub>5</sub>*) and 0.30 (P-450) and for the nuclear membranes as 0.026  $\mu\text{M}/\text{mg}$  protein (*b<sub>5</sub>*) and 0.032 (P-450), respectively. With rat liver material, *b<sub>5</sub>* contents of 0.13 (microsomes) and 0.034 (nuclear membranes) and P-450

contents of 0.18 (microsomes) and 0.025 (nuclear membranes) were found. Our microsomal values thus lie in the same range as those reported from other mammalian liver microsomes by various authors (e.g. 20, 21, 51, 66, 67).

The fact that the enzyme activities determined in this study are in general of the same order of magnitude as those found by other authors using different isolation procedures indicates that neither the special medium components used (cf. reference 46) nor the inevitably lengthy preparative procedures (in some experiments up to 30 hr) lead to a considerable loss of enzyme activities. This is also suggested by the coincidence of the results obtained from microsomes prepared by using our isolation medium ([*i*] of Materials and Methods) with those prepared according to a reference (*ii*).

Measurements on the activity of cytochrome oxidase, another enzyme presumed to be an integral part of the mammalian liver nuclear envelope (47, 97), have not so far yielded clear-cut results. There is, however, considerably less activity present than in the preparations of other authors.

#### Gel Electrophoresis

Results of the polyacrylamide gel electrophoresis of nuclear membranous and microsomal material from pig liver are presented in Fig. 6. The pattern of the membrane moiety which could be solubilized with the 6 mM mercaptoethanol (pH 9.0) is shown at the left. With the nuclear membranes, about 20 bands could here be detected, six of which appear as main bands, while the microsomal material revealed nearly 24 bands, including some barely visible, minor ones. At least twelve proteins appear to be identical in both samples. An interesting feature observed by comparing band patterns is that the intensities of some common bands are different for the two kinds of membranes. Preliminary experiments in which urea and detergents were omitted yielded very few (1-3) bands from this mercaptoethanol-solubilized material, an observation which is in agreement with the findings by Berkman et al. (7) on the proteins of rat liver microsomes.

When the other moiety of the two dialyzed membrane fractions, which is sedimentable with 100,000g and which constitutes about 80% of the total protein of the whole fractions, was solubilized with phenol-urea-acetic acid and applied to the gel system of Takayama et al. (88), 11 proteins migrated into the gel and produced visible

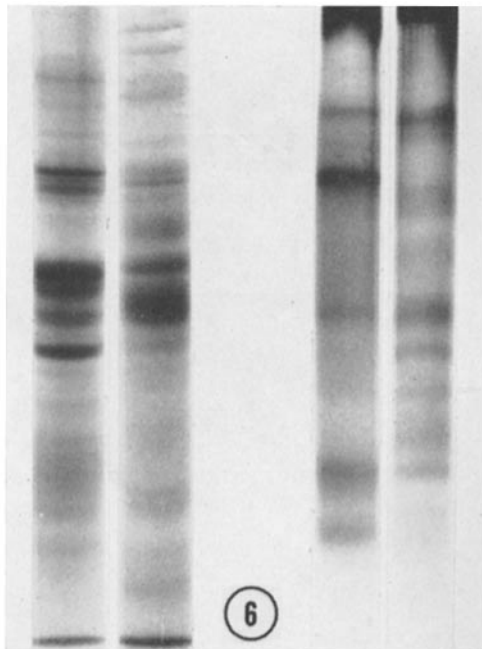


FIGURE 6 Gel electrophoresis patterns of microsomal and nuclear membrane proteins. *Left pair*, separation of proteins which could be solubilized from the membranous fractions during dialysis with 6 mM mercaptoethanol at pH 9.0; besides a great many common bands (*circa* 12), a few proteins distinct for either microsomes (left gel) or nuclear membranes can be recognized; gel system No. 1 of reference 53. *Right pair*, membrane material sedimentable (100,000 g) after the dialysis with 6 mM mercaptoethanol. When prepared according to Takayama et al. (88), this membrane protein moiety reveals, besides a general correspondance (*circa* 15 common bands), at least four additional proteins for the nuclear membrane material (right trace of the pair) in comparison with the microsomal pattern. Note that here apparently not all the material has entered the gel.

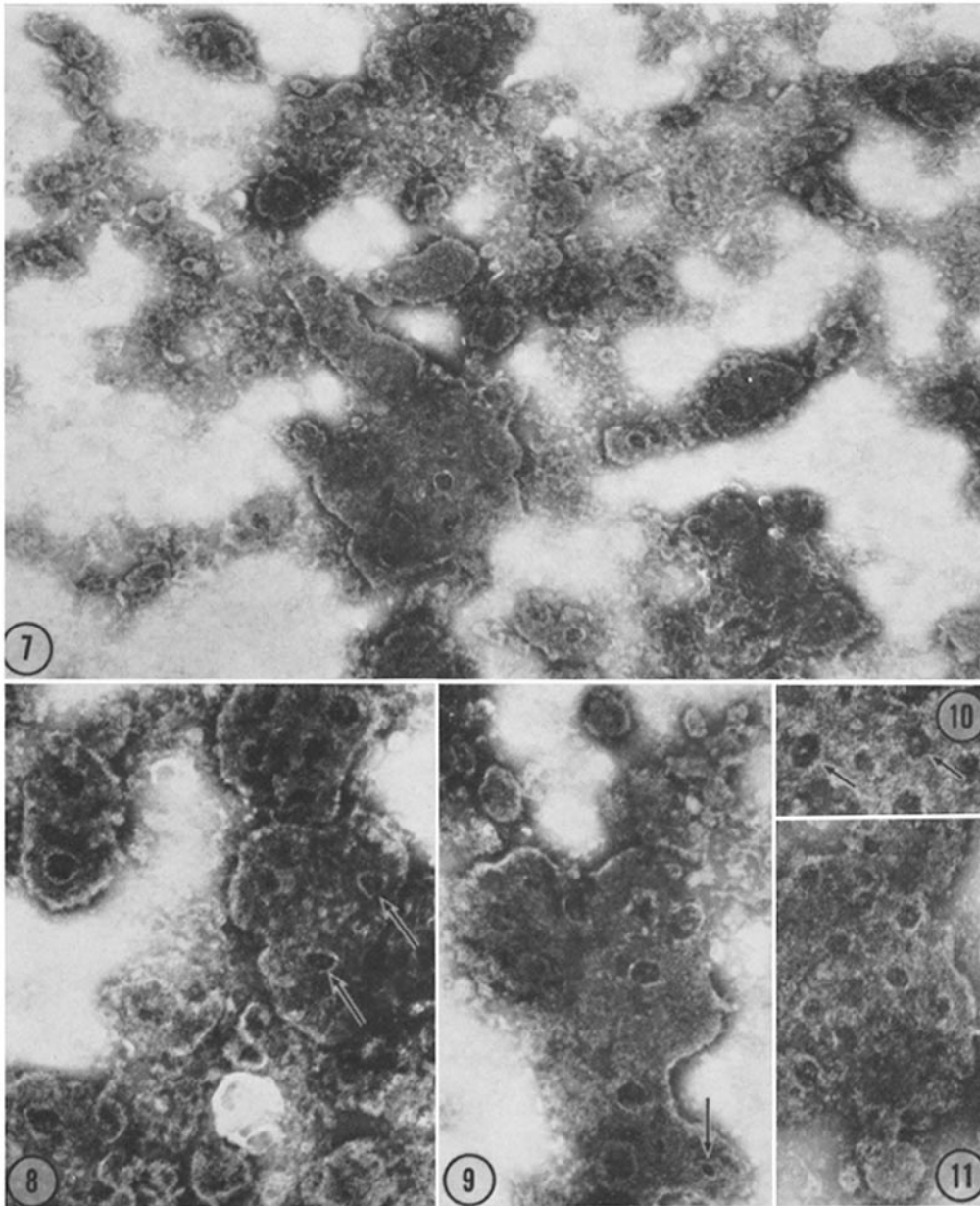


FIGURE 7 Survey of a nuclear membrane fraction isolated from pig liver, negatively stained with phosphotungstate without prior fixation. Note the varying size of the fragments obtained. A remarkable amount of the membrane material has been fragmented into very small particles.  $\times 42,000$ .

FIGURE 8 Preparations as in Fig. 7. The characteristic nuclear envelope fragments show marked alterations in the nuclear pore shape (arrows). Central inner pore material appears to be present in many of the pores.  $\times 56,000$ .

FIGURE 9 Besides the pores, the envelope fragments sometimes exhibit small breakage holes (arrow) presumed to be artificially caused during the isolation.  $\times 60,000$ .

FIGURE 10 Similar preparation from rat liver; note the typical central granules in the pores (arrows).  $\times 65,000$ .

FIGURE 11 Partially degraded nuclear envelope fragment with the pore complexes still widely intact.  $\times 56,000$ .

bands. Five of these bands were common to both microsomes and nuclear membranes. It is noteworthy that, except for only one band, all microsomal protein bands had corresponding bands in the nuclear membrane pattern, but that, in the latter, four additional proteins are obviously present. It should, however, be emphasized that a considerable amount of this "sedimentable" material did not enter the gel with the solubilization procedures so far applied. The observed differences in the protein band pattern have meanwhile been confirmed by split-gel comparisons, too (B. Deumling, 1970, manuscript in preparation).

#### *Morphology of the Nuclear Membrane Fraction*

In agreement with Kashnig and Kasper (43) our experience indicates that sonication of the nuclei is the most crucial step affecting the size of

the envelope fragments obtained. In general, we found that the banding in the continuous membrane gradient was much sharper and faster if the fragmentation procedure was relatively vigorous. The sizes of the fragments in our fractions varied over a wide range (from *circa*  $2 \times 10^{-4} \mu^2$  to about  $1 \mu^2$ ) as can be seen in the survey micrographs of negatively stained (Figs. 7-12) as well as of sectioned preparations (Fig. 13). With negative staining, larger nuclear envelope fragments, including some with up to 10 pore complexes, could be recognized in addition to smaller ones which showed few or no pores. Membrane material that was further disaggregated to smaller components, including various globular ring-shaped or more filamentous structures, was also consistently observed. A somewhat surprising result was the finding that even after the high salt treatment applied, nonmembranous pore complex constituents (28, 30) such as the central granule and the inner pore

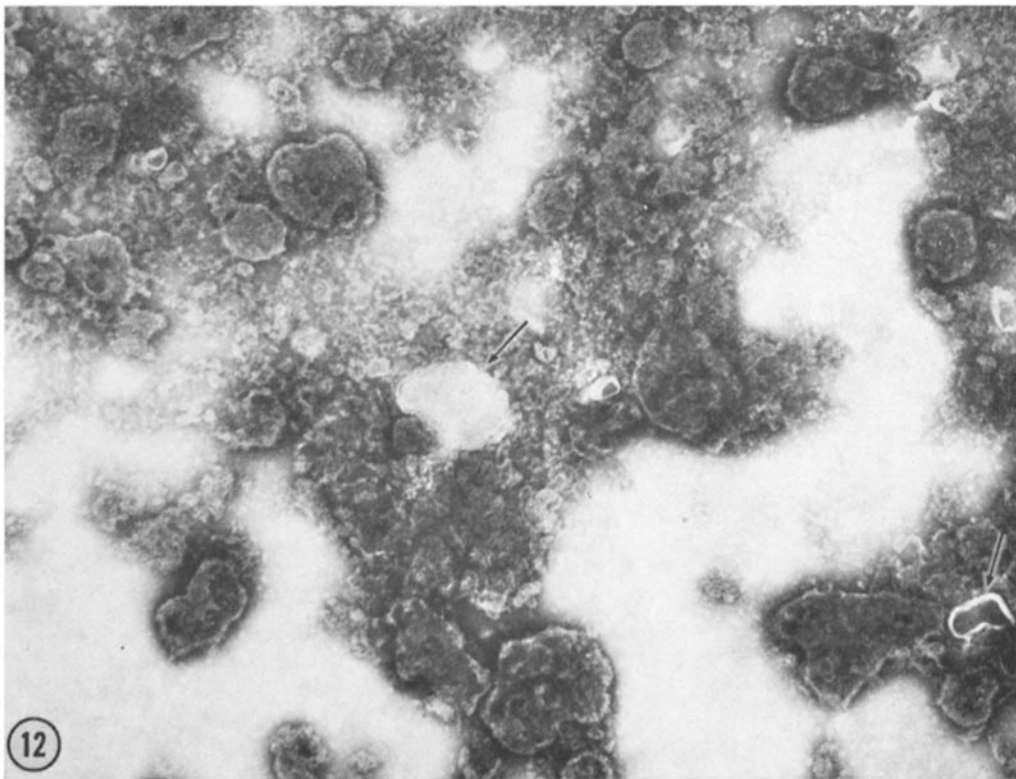
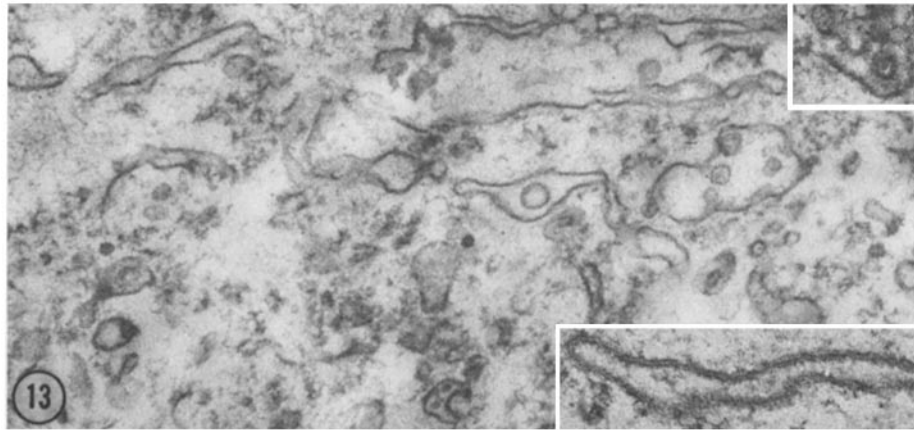


FIGURE 12 Nuclear membrane fraction from pig liver, fixed with  $\text{OsO}_4$  prior to the negative staining with phosphotungstic acid. No structural alterations are obvious in comparison with the nonfixed membrane fractions of the previous micrographs. Note that also after this type of preparation some myelinization can be observed (arrows).  $\times 43,000$ .



**FIGURE 13** Thin section through nuclear membrane material isolated from pig liver after fixation with glutaraldehyde plus  $\text{OsO}_4$ . Note the varying size of the membranous particles. Upper *insert* shows tangentially sectioned pore complex with annulus and central granule; lower *insert* presents the trilamellar unit membrane structure of such membranes.  $\times 42,000$ ; upper *insert*  $\times 60,000$ ; lower *insert*,  $\times 90,000$ .

fibrils could often still be identified (e.g. Figs. 8, 10, 12), a finding which is in obvious contrast to the work by Mentré (60). Annular particles, however, were only rarely observed in such preparations (Figs. 7–13). The mean pore diameter seemed to be slightly smaller (*circa* 500 Å for pig and 540 Å for rat material) after the sonication and extraction procedures than before. Deviations from the circularity of the pores were frequent, including chiefly ellipsoidal and polygonal shapes (Fig. 8). No differences were apparent between fractions fixed with  $\text{OsO}_4$  prior to the negative staining procedure and such which were directly used without any fixation (Figs. 7, 12). This indicates that no considerable additional membrane degradation takes place due to the influence of the phosphotungstic acid or to the spreading and drying in the negative staining process. Obvious artificial structures, however, were small “crack holes” (Fig. 9; cf. also references 15, 30, 77) and typical myelinization figures (e.g. Fig. 12). No differences were apparent between fractions from pig and rat liver, except that the myelinated material appeared to be more frequent in the former. The appearance in thin section corresponded to that of the negative staining. The envelope fragments appeared either as closed cisternae including pores or as vesicles or, in consequence of a further-reaching degradation as single membrane sheets (Fig. 13). A triple-layered “unit membrane” structure, rarely seen in

nonisolated nuclear and ER membranes (cf., e.g., reference 37), was generally resolved within the 70–90 Å broad nuclear membranes (Fig. 13; cf. also references 26, 43). In sections grazing to nuclear envelope fragments the pore was sometimes characterized by an electron-opaque annulus and the centrally located pore granule (Fig. 13). The nuclear pores appeared in cross-section to be either “filled” with inner pore material or “open,” in both cases often exhibiting remnants of the annular constituents lying attached to the pore margin. 15–30 Å thin filaments intimately associated with the nuclear membranes could represent partially degraded membranous protein or the above-mentioned nucleoprotein which remained in close attachment to the membrane throughout the high salt solubilization. The latter interpretation seems to be particularly supported by their relatively high stainability with uranyl acetate.

#### DISCUSSION

In the last 3 yr attempts have been made in different laboratories to isolate masses of nuclear membrane material from liver, starting from purified nuclear fractions. The methods used for isolating the nuclei were modifications of the Chauveau procedure which utilizes sucrose media with or without the addition of bivalent cations in millimolar concentrations. Fragmentation of the iso-

lated nuclei was accomplished with osmotic shock with or without subsequent sonication (26, 43, 97). In some investigations nucleoproteins adhering to the nuclear envelope membranes were removed by dissolving them with decomplexing agents such as citrate which was applied either in acid (9, 83) or slightly alkaline (43) solutions. Our method represents an alternative approach in which isolation media slightly modified from those introduced by Kuehl (45) were used for the nuclear isolation, in which isotonic conditions were maintained throughout the whole procedure and a high concentration of KCl was applied to solubilize the nucleoproteins (cf. the review in reference 34). Summarizing the results of the present study and those of the other groups, one now is able to give a first characterization of mammalian liver nuclear membranes.

Our nuclear membrane fractions are characterized by a very low RNA:protein ratio which is of the same order of magnitude as that of the microsomes prepared in parallel with them. Furthermore, a certain amount of DNA also remains associated with the nuclear membrane fragments (cf. also 9, 97). From the chemical point of view the similarity between microsomal and nuclear membranes is striking, in particular if the data are compared with plasma membrane data from the same tissue. Both kinds of cytomembranes as defined by Sjöstrand (recently reviewed, e.g. in references 73 and 82) are equal in cholesterol content (44), in their contents of nonpolar lipids, and have nearly identical phospholipid patterns (44). Furthermore, they have several membrane proteins in common, as is apparent from the gel electrophoresis and the enzyme tests performed. Some enzymes, including Mg-ATPase and both NADH- and NADPH-cytochrome *c* reductase, which typically occur in microsomes, apparently are present in the nuclear membranes as well. Data in the literature on their relative activities in nuclear versus microsomal membranes are contradictory (cf. references 43 and 97) and hitherto not conclusive. Kashnig and Kasper (43) discussed activity differences of *circa* 50% in glucose-6-phosphatase and NADH-cytochrome reductase as an indication that these enzymes would be associated with only one of the nuclear envelope membranes, presumably the outer one. However, any conclusions derived from such relative enzyme activity differences between microsomes and nuclear membranes are hazardous, especially if one

considers the divergent results of different groups and keeps in mind that disproportional masking and inactivation can occur and that the apparent enzyme activities of membrane fractions depend to a considerable degree on the extent of membrane fragmentation, i.e., the size of the particles suspended in the assay.

On the other hand, there exist some significant differences between microsomes and nuclear membranes. We found nuclear membranes to be distinct from microsomes as well as from Golgi (62) and plasma membranes, in that they are richer in protein. This is especially apparent if one takes into account possible adhering nucleoproteins in calculating membrane phospholipid:protein ratios. Such a maximum calculation proceeds from the assumption that the protein moiety of the membranous fraction, which is complexed to nucleic acids, is present in the same relative w/w ratios characteristic for ribosomal material (in the case of RNA) and chromatin (if DNA is present). After subtraction of the thus calculated "non-membrane" protein moiety, the phospholipid:protein value of the nuclear membranes is significantly below that of the microsomal membranes of our study as well as of the different microsomal fractions of other laboratories (Table II; for guinea pig cf. also reference 69). This higher protein content is likely to contribute to the higher density of the nuclear membrane fragments. While differences in the lipid compositions are minor (44), differences in proteins were found, besides many common bands, in the gel electropherograms (Fig. 6). We would speculate that some of the additional nuclear membrane proteins represent pore complex material. On the other hand, a few proteins present in the microsomes are almost totally absent in the nuclear envelope, e.g., the monovalent cation-stimulated ATPase and the glucose-6-phosphatase. With respect to this latter enzyme, our data are in agreement with the data of Zbarsky et al. (97) but are in contrast to those of Kashnig and Kasper (43) who reported a reduced but still considerable glucose-6-phosphatase activity in their nuclear membrane fraction. They are also in contrast to earlier histochemical work which suggests the existence of this enzyme in the nuclear envelope (e.g. 36, 90). An inactivation of the glucose-6-phosphatase activity in the nuclear membrane fractions during the preparation might be indicated by the differences between the nuclear fractions and the membranes prepared

therefrom. On the other hand, this seems to be unlikely since the microsomes which had been treated in the very same way showed high activity. Our inhibition experiments, moreover, strongly suggest that what we have measured is actually specific glucose-6-phosphate phosphohydrolysis (Table V). Thus we are, in accordance with Zbarsky's people (97), inclined to conclude that glucose-6-phosphatase which is a marker enzyme for microsomes is not present in nuclear membranes and could therefore serve as a marker for monitoring microsomal contamination in nuclear membrane preparations. Very low glucose-6-phosphatase activities are also known, among the liver cytomembranes, for Golgi membranes (25, 61, 62).

It is noteworthy that, taken together, the membranes of the endoplasmic reticulum and those of the nuclear envelope, i.e. membranes which are known to be in morphological continuity, and to have also many common components, show characteristic differences with respect to some constitutional proteins. Interspecific differences between rat and pig material were so far not observed.

It is also worth emphasizing that some RNA as well as some DNA remains attached to the membranes. The RNA in the nuclear membrane fractions may represent remnants of the ribosomal and nonribosomal RNP's attached to the outer membrane (cf., e.g., reference 83) as well as represent true "membrane-RNA" which seems to be a constituent of diverse cellular membranes (review, e.g. references 63, 80, 91, 96). Furthermore, some RNA associated to nuclear membrane fragments may represent elements of the nuclear pore complex material such as central granules, inner fibrils, and annulus constituents which are widely presumed to be of RNP nature (28, 30, 31, 60, 76, 86). Since these structures, especially the central granules, apparently withstand the preparation

procedures, including the high salt solubilization, it appears not unlikely that such pore complex-associated RNA could contribute to the total nuclear membrane-associated RNA. The observation that a certain amount of DNA also sticks to the nuclear membrane, presumably the inner leaflet, is consistent with the suggestions of various authors (e.g. 3, 16, 17; see, however, also reference 71) who indicate that the inner nuclear membrane may be involved in starting and regulating events in the reduplication of the nuclear DNA. Thus, the functional nuclear membrane-DNA associations may be analogous to those of the Jacob concept for bacterial DNA replication (e.g. 41, 42, 55). Details of the nuclear membrane-associated nucleic acids will be communicated in one of the next contributions of this series.

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*Note Added:* After completing this manuscript, we learned of the paper given at the Detroit meeting of the American Society of Cell Biology by R. Berezney, L. K. Funk, and F. L. Crane (1969, *J. Cell Biol.* 43:12a) on nuclear membranes isolated from bovine liver by a method different from ours. The results of these authors agree widely with those of the present study on pig and rat liver material. This holds in particular for the relatively high protein content, the low glucose-6-phosphatase activity, the amount of Mg-ATPase activity, and the low content of P-450. Values for the activity ratios of NADH- and NADPH-cytochrome *c* reductases differ from ours.

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