The Purification and Biochemical Characterization of Bovine Liver Nuclear Membranes

(Received for publication, September 2, 1971)

RONALD BEREZNEY,† LINDA K. MACAULAY, AND FREDERICK L. CHAND§

From the Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

SUMMARY

Bovine liver nuclear membranes were purified by means of a high salt (0.5 M MgCl₂) discontinuous sucrose gradient. The purified nuclear membranes were isolated at a density of ρ = 1.05 to 1.23 and contained 62% of the protein recovered from the gradient. The presence of nuclear pure complexes identified the fraction as nuclear membrane. Twenty-six percent of the protein was recovered at a density of ρ = 1.23 to 1.30. Electron microscopy demonstrated both nuclear membranes and nonmembranous nucleolar components with a predominance of the latter. A fraction sedimenting through the most dense high salt sucrose solution (ρ ≥ 1.30) made up 12% of the total recovered protein. Ultrastructural analysis revealed nucleolar constituents in both a granular fibrillar network and in the more condensed form characteristic of nucleoli in intact nuclei. In the absence of high salt, the nuclear membranes and nucleolar material aggregated and sedimented at a density intermediate between the lighter membranes and the heavier nonmembranous components.

Purified nuclear membranes consisted of 70.4% protein, 5.8% RNA, 1.1% DNA, and 22.6% phospholipid. The comparatively nonmembranous nature of the heavier fractions was indicated by the much lower phospholipid contents of 9.7% and 9.8%, respectively. Since nucleoli have a characteristic high RNA content, the much higher RNA levels of the heavier fractions (8.0% and 11.4%), as compared to the lighter nuclear membranes, were consistent with the ultrastructural demonstration of nucleolar components in these fractions.

Eighty to 90% of the endoplasmic reticulum-type enzymes, α-NADH-cytochrome c reductase, glucose-6-phosphatase, and Mg⁺⁺-stimulated ATPase, and the characteristic inner mitochondrial membrane activity, cytochrome c oxidase, were not due to microsomal and mitochondrial contamination. The low succinate dehydrogenase activity in purified nuclear membranes is correlated with the absence of acid nonextractable, trypsin-releasable flavin, which is associated with mitochondrial succinate dehydrogenase flavoprotein. In addition, coenzyme Q, a characteristic component of inner mitochondrial membranes, was not detected. The above enzyme activities were increased 1.2- to 1.9-fold in purified nuclear membranes compared to the unpurified membranes. In contrast, the high salt sucrose gradient fractions of higher density demonstrated progressive decreases in specific activities from 0.32 to 0.61 in the intermediate density fraction to 0.15 to 0.41 in the heaviest fraction. This was paralleled by the progressive decreases in nuclear membranes in these two fractions. Moreover, 84 to 90% of the total enzyme activities were found in the purified nuclear membranes, whereas only 7 to 15% and 1 to 4% were present in the intermediate and heaviest fractions, respectively.

The membranes contained 0.322 nmole of cytochrome b₁ per mg of protein and 0.045 nmole of cytochrome aₐ₃ per mg of protein, which were 33% and 30% of values in microsomes and mitochondria. The cytochrome P-450 content of nuclear membranes was only 4.4% of microsomes and can be attributed to microsomal contamination.

Direct enzymatic comparison of nuclear and microsomal membranes demonstrated a strong endoplasmic reticulum-type character in nuclear membranes. Nuclear membranes differed from endoplasmic reticulum, however, by the presence of cytochrome c oxidase activity and cytochrome aₐ₃ and the only trace amounts of NADPH-cytochrome c reductase and cytochrome P-450.

An earlier communication from this laboratory (1) demonstrated that certain enzyme activities characteristic of endoplasmic reticulum membranes, as well as a mitochondrial type enzyme activity, cytochrome c oxidase, were endogenous to bovine liver nuclei. The investigations reported herein are concerned with the specific localization of these enzyme activities in morphologically recognizable nuclear structures, as well as with the characterization of nuclear electron transport activity.

This first paper is concerned with the further purification and biochemical characterization of nuclear membranes. Previously reported results (2) indicated that the nuclear membrane fraction was highly purified with respect to other cellular membranes, but was contaminated with nuclear-derived structures. Since the enzymes investigated are typically membrane bound, it is reasonable to conclude that the nuclear activities are associated...
with the nuclear membrane. Although such a conclusion is consistent with the current state of knowledge in membrane biochemistry, it cannot be assumed a priori, particularly in light of the reported concentration of nuclear electron transport components in so-called nucleoli fractions (3-5). A necessary step for conclusively establishing the site of nuclear enzyme activities is therefore the separation of the nuclear membranes from the contaminating nucleolar components. A portion of these studies have appeared elsewhere in preliminary form (6, 7).

MATERIALS AND METHODS

Isolation of Nuclei

A scheme for the preparation of bovine liver nuclei on a large scale is presented in Fig. 1 and represents a modification of a previously reported procedure (2). A bovine liver weighing a minimum of 10 pounds was obtained immediately after the animal had been sacrificed. Subsequent to the removal of the outer covering membrane and excess connective tissue, the liver was cut into approximately 1-cm³ cubes, and immediately placed into a solution of ice-cold sucrose Buffer A (0.05 M Tris-HCl, pH 7.5, 0.25 M sucrose, 0.025 M KCl, 0.005 M MgCl₂ (8)). The liver cubes were washed several times in sucrose Buffer A and homogenized in a Waring Blender in the ratio of 1 volume of liver to 3 volumes of sucrose Buffer A at speeds ranging from 12,000 to 13,500 rpm for periods of 30 to 60 s. The actual speed and time depended on the consistency of the particular liver, e.g. very tender livers were blended for 30 s at 12,000 rpm, whereas tougher livers were blended up to 60 s at 13,500 rpm. After filtration through 2, 4, and 8 layers of cheesecloth in rapid succession, the homogenate was centrifuged in a high capacity (13 liter) International centrifuge at 2,000 rpm (1,800 × g) for 20 min.

The crude nuclear pellets were washed 2 times with sucrose Buffer A and centrifuged at 800 × g for 5 min in an International centrifuge equipped with a 4-liter capacity swinging bucket rotor. After resuspending the pellets in 2.3 M sucrose Buffer A and filtering through eight layers of cheesecloth 2 times, the volume was adjusted from 2,500 to 5,000 ml (depending on the size of the pellets), and the suspension was centrifuged at 78,480 × g for 20 min in the Spinco 30 rotor. To collect the nuclei, the centrifuge tubes were inverted and visible contamination removed with a spatula and by swabbing with tissue paper. The surface of the nuclei pellet was rinsed a few times with sucrose Buffer A, resuspended in the same buffer, and centrifuged at 780 × g for 5 min in the SS-34 rotor of the Servall centrifuge.

For final purification, the nuclear suspension was layered on a discontinuous sucrose gradient according to the procedure of Blobel and Potter (8), as adapted by Kashig and Kasper (9) for larger scale preparations. Two volumes of 2.3 M sucrose Buffer A were added to 1 volume of nuclear suspension, and 40 ml of this was layered on top of 15 ml of 2.2 M sucrose Buffer A and centrifuged at 25,000 rpm (75,500 × g) for 30 min in the Spinco SW 25.2 rotor. The purified nuclei at the bottom of the tube were washed in sucrose Buffer A, centrifuged at 780 × g for 5 min, and resuspended in sucrose Buffer A.

Preparation of Mitochondria and Microsomes

Mitochondria and microsomes were prepared from the supernatant of the first centrifugation. Mitochondria were isolated by centrifuging the supernatant at 4,000 × g for 15 min. The supernatant of the second centrifugation was centrifuged at 27,000 × g for 15 min, and the pellets discarded. The third supernatant was centrifuged at 104,000 × g for 1 hour to isolate the microsomes. Both the mitochondrial and microsomal fractions were washed 2 times by resuspending in sucrose Buffer A and centrifuging the mitochondria at 4,000 × g for 15 min and the microsomes at 104,000 × g for 1 hour.

Preparation of Total Nuclear Membrane Fraction

The suspension of purified nuclei (2 mg of protein per ml) was digested with deoxyribonuclease (DNase I) at a concentration of 50 µg of DNase per ml for 10 to 14 hours at 2°. After the digestion period, the nuclei were washed 2 times with sucrose Buffer A, centrifuged at 780 × g for 5 min, and resuspended in 0.5 M MgCl₂ sucrose Buffer A. This high salt suspension was immediately centrifuged at 104,000 × g for 30 min in the Spinco 40 rotor. The pellet, representing the total nuclear membrane fraction, was washed first with 0.5 M MgCl₂ sucrose Buffer A, and then with sucrose Buffer A at identical centrifugations of 104,000 × g for 30 min, and finally resuspended in a small amount of sucrose Buffer A for subsequent analysis. For comparative studies mitochondria and microsomes isolated above were treated with DNase and MgCl₂ exactly as nuclei.

Sucrose Gradient Purification of Nuclear Membrane Fraction

Discontinuous sucrose gradients were prepared by layering 15 ml of the appropriate buffer which was 1.6 M in sucrose on top of 15 ml of 2.2 M sucrose solution. Two different gradients were prepared and run. The first consisted of sucrose in Buffer A and the second, sucrose in Buffer A made 0.5 M in MgCl₂ (high salt sucrose gradient). Twenty-five ml of the total nuclear membrane fraction at a protein concentration of 1 mg per ml was layered on top of the appropriate gradient systems and centrifuged for periods ranging from 30 min to 20 hours in the SW 25.2 Spinco rotor at 25,000 rpm. For smaller scale experiments the SW 39 rotor was used and the volumes reduced accordingly.

Fractions were collected with a syringe and needle, the tip of which was bent to form a 90° angle. Fig. 2 illustrates the frac-

Liver homogenate

[1,800 × g, 20 min]

Crude nuclear pellet

(1) Suspend in sucrose TKM
(2) 800 × g, 5 min
(3) Discard supernatant
(4) Repeat Steps 1 to 3

Washed crude nuclear pellet

(1) Suspend in 2.3 M sucrose TKM
(2) 78,480 × g, 20 min

Nuclear pellet

(1) Suspend in sucrose TKM
(2) 780 × g, 5 min
(3) Discard supernatant
(4) Repeat Steps 1 to 3

Washed nuclear pellet

(1) Suspend in sucrose TKM
(2) Add 2 volumes of 2.3 M sucrose TKM
(3) Layer on top of 2.2 M sucrose TKM
(4) 75,500 × g, (SW 25.2), 30 min
(5) Resuspend pellets in sucrose TKM
(6) 780 × g, 5 min
(7) Resuspend in sucrose TKM

Purified nuclei

Fig. 1. Scheme for the preparation of bovine liver nuclei. TKM, Buffer A.
tions obtained. Material collected at the 0.25 m to 1.6 m sucrose interface is referred to as 1.6I, that at the 1.6 m to 2.2 m interface is labeled 2.2I, and any material which centrifuges through the 2.2 m sucrose is referred to as 2.2P.

The sucrose gradient centrifugation described above resulted in the development of a procedure for the further purification of nuclear membranes. The scheme for preparation is outlined in Fig. 3. Further details will be presented under "Results."

Chemical Determinations

Protein—Protein content was estimated by the procedure of Lowry et al. (10), or by a modified biuret reaction (11).

Nucleic Acids—RNA and DNA were separated as described by Munro and Fleck (12). DNA was determined by the diphenylamine reaction (13) with calf thymus DNA as a standard. RNA content was determined spectrophotometrically according to Munro and Fleck (14). For calculation an absorbance of 1.000 equaled 32 µg of RNA per ml at 260 nm.

Lipid—Lipids were isolated by the method of Folch et al. (15), and phosphorus analysis was performed according to Cheu et al. (16). The phospholipid content in micrograms was calculated by multiplying the determined number of micrograms of phosphorus by 25.

Coenzyme Q (Ubiquinone)—A spectrophotometric assay was employed in which the reduction of coenzyme Q with borohydride was determined according to the procedures of Crane and Dillye (17).

Flavin—Acid-extractable and acid-nondeoxidable flavin were assayed according to the methods of Blair et al. (18).

Cytochrome b₄—Reductions for difference spectra were achieved by addition of a few grains of dithionite. In some cases reduction was achieved with NADH in the presence of 1 mM KCN. A base-line was obtained by recording the oxidized versus the oxidized spectrum. Cytochrome b₄ was estimated from the reduced versus oxidized difference spectrum at 426 to 410 nm by means of an extinction coefficient of 160 cm⁻¹ m̅mol⁻¹ (19).

Cytochrome P₄₅₀—Difference spectra for cytochrome P₄₅₀ were obtained by measuring the difference between the sample reduced with dithionite and treated with carbon monoxide versus the sample reduced with dithionite. The reduction and carbon monoxide treatment were performed under anaerobic conditions by means of Thunberg cuvettes. An extinction coefficient of 01 cm⁻¹ m̅mol⁻¹ at 450 to 460 nm was used to calculate P₄₅₀ (20).

Cytochrome aₐₙ—In order to measure the a-band of cytochrome aₐₙ, a protein concentration of 9.0 mg per ml was used, and turbidity reduced by the addition of 0.05 ml of 10% deoxycholate (sodium salt) per ml of particle suspension. An extinction coefficient of 13.1 cm⁻¹ m̅mol⁻¹ at 605 to 630 nm was used in calculating cytochrome aₐₙ content (21). All spectral recordings were obtained on a Shimadzu MSP-50L recording spectrophotometer.

Enzyme Assays

NADH, Succinate, and NADPH-Cytochrome c Reductases—Activity was assayed by following the reduction of cytochrome c at 550 nm according to Ernst et al. (22). The assay mixture, in a total volume of 3 ml, contained 0.05 M Tris-HCl, pH 7.5, 0.33 mM KCN, 0.05 mM cytochrome c, 0.100 ml of the appropriate particle suspension, and 0.1 mM NADH, NADPH, or 5.0 mM sodium succinate (pH 7.0).

NADH-Ferricyanide Reductase (NADH Dehydrogenase)—Ferricyanide reduction was followed at 420 nm. The 3-ml assay mixture contained 2.0 mM ferricyanide, 0.33 mM KCN, 0.100 ml of the appropriate particle suspension, 0.2 mM NADH, and 0.05 M Tris-HCl, pH 7.5. The rate in the absence of enzyme was subtracted from the enzymatic rate.

Succinate Dehydrogenase—Activity was measured by measuring the reduction of phenazine methosulfate. The assay was performed spectrophotometrically at 600 nm to determine 2,6-dichlorophenolindophenol reduction by the reduced phenazine methosulfate as described by King (23) with the use of a fixed phenazine methosulfate concentration of 0.3 mM. Monoamine oxidase was determined as by Schmitman et al. (24).

All spectrophotometric assays were performed at 30° on a Unicam model SP-800 spectrophotometer equipped with a Bausch and Lomb external recorder in which the absorbance scale was expanded either 10- or 20-fold.

Glucose 6-Phosphatase and Mg⁺⁺stimulated ATPase—Activity was assayed by measuring the amount of inorganic phosphate

Fig. 2. Fractionation of the total nuclear membrane fraction on sucrose gradients. The total nuclear membrane fraction was separated on a discontinuous gradient. Three possible fractions can be obtained. 1.6I corresponds to material at the 0.25 m to 1.0 m sucrose interface; and 2.2I represents material centrifuging through 2.2 m sucrose.

Fig. 3. Outline for the preparation of purified nuclear membranes. TKM, Buffer A.
released after 10 min at 30°C according to the procedures of Swan-
son (25) and Ernster et al. (22), respectively. Inorganic phos-
phate was determined by the isobutyl alcohol-benzene extraction
procedure of Martin and Doty (16) as modified by Lindberg and
Ernster (27). A standard curve was used in which the optical
density at 1 mg was 0.385 μmoles of Pi per ml. Readings were
taken on a Beckman DU spectrophotometer.

**NADH Oxidase, Succinoxidase, and Cytochrome c Oxidase**—
These activities were measured polarographically at 37°C with a
Gilson KM oxigraph equipped with a Clark electrode covered
with a Teflon membrane. For NADH oxidase the assay con-
tained 66.7 μmoles of phosphate at pH 7.5, cytochrome c (2 mg
for the mitochondrial assays and 8 mg for nuclear and microsomal
assays), and the appropriate particle suspension in a volume of
1.6 ml. The reaction was followed for approximately 3 min, then
0.2 ml of 1% NADH was added. The NADH oxidase rate was
obtained by subtracting the rate before NADH was added from
the rate in the presence of NADH.

Succinoxidase assays were performed in the same manner
except that 2 mg of cytochrome were added for both nuclear and
mitochondrial fractions.

Cytochrome c oxidase was assayed by a modification of the
polarographic procedure of Chuang et al. (28). The assay mix-
ture consisted of 16 mM potassium phosphate, 10 mM potassium
citrate, 0.80 mM EDTA, 13 mM potassium ascorbate (pH 6.5),
1.11 mM N,N,N',N'-tetramethyl-p-phenylenediamine di-
hydrochloride, and 20 mg cytochrome c in a total volume of 1.6
ml. After recording an initial rate, the particle suspension was
added. The enzymatic rate was calculated by subtracting the
rate in the absence of added enzyme from the rate in its pres-
ence.

**Enzymatic Evaluation of Mitochondrial and Microsomal Con-
tamination and Their Contribution to Enzyme Activities in Nuclear
Fractions**—Mitochondrial contamination was estimated by as-
suming that succinate dehydrogenase activity is exclusively mi-
tochondrial (29). The percentage of mitochondrial protein in
nuclear fractions was calculated by comparing their succinate de-
hydrogenase activities with the mitochondrial values. The per-
centage of microsomal protein contamination was measured in a
similar manner with NADPH-cytochrome c reductase as a
marker for microsomal membranes (30).

In order to calculate the contribution of mitochondrial or mi-
crosomal contamination to a particular enzyme activity in a
nuclear fraction, the percentage of protein contamination was
multiplied by the activity of the particular enzyme in the mitc-
chondria or microsomes.

Corrected values for enzyme activities in nuclear fractions
were obtained by subtracting these contamination activities.

**Electron Microscopy**

Specimens prepared for sectioning were pre-fixed with 2.5%
formaldehyde buffered in 0.1 M potassium phosphate (pH 7.4)
at 0°C, and postfixed in 1% osmium tetroxide in an acetate-ver-
onal buffer according to Palade (31). Samples were dehydrated
in acetone and embedded in Epon based on a procedure by Luft
(32). Sectioned specimens were poststained with 0.2% lead
citrate in 0.1 M NaOH for 3 min at room temperature. Samples
negatively stained with 2% phosphotungstic acid at pH 7.0 were
prepared according to the method of Cunningham and Crane
(33), and were observed and photographed with a Philips EM
300.

---

**Chemicals**

NADH, NADPH, cytochrome c, ATP, ADP, succinic acid,
bovine serum albumin, Trizma base, and phenazine methosul-
fate were obtained from Sigma Chemical Co. Glucose 6-phos-
phate, calf thymus DNA, and deoxyribonuclease I was purchased
from Calbiochem. N,N,N',N'-Tetramethyl-p-phenylenedia-
mine dihydrochloride was the product of Eastman Organic Chem-
ical Corporation. All other chemicals were either reagent grade
or of the highest available purity.

**RESULTS**

**Nucleolar Contamination in Nuclear Membrane Fraction**—Nu-
clear material contaminating the total nuclear membrane fra-
tion is shown in Fig. 4. The granular-fibrillar appearance of
this material is due to the effect of high salt treatment on the
structure of nucleoli. Both Ris (34) and Georgiev (35) have
reported that extraction with high salt converts the typical con-
densed form of nucleoli to a more granular-fibrillar appearance.
The condensed form of nucleoli with the characteristic lacunae
(see References 35 and 36) were also observed in NMF.

The procedure for nuclear membrane isolation involved cen-
trifugation after high salt extraction of DNase-treated nuclei
(see "Materials and Methods"). Differential separation of nu-
cleolar components from nuclear membrane was not successful.
At low speeds (1000 × g, 5 min), both large fragments of nuclear
membranes and relatively intact nucleoli sedimented. At higher

---

1 The abbreviations used are: NMF, total nuclear membrane
fraction; ER, endoplasmic reticulum.
Fractionation of NMF on Sucrose Gradients—Attempts at purifying NMF by conventional sucrose gradients proved only partially successful. Fractions were centrifuged in gradients prepared in sucrose Buffer A for periods ranging from 30 min to 20 hours. Protein (70.8%) banded at the 1.6 to 2.2 M sucrose interface which is referred to as 2.2I (Fig. 5). Analysis of this 2.2I fraction showed no appreciable differences from NMF in either structure or enzymatic activities. Electron microscopic examination of the lighter 1.6I fraction (0.25 to 1.6 M sucrose interface), however, revealed the concentration of nuclear membranes. Unfortunately the fraction represented less than 10% of the total protein. The pellet which centrifuged through 2.2 M sucrose, 2.2P, was highly concentrated in nonmembranous components. This partial separation was indicative of an inherent density difference between nuclear membranes and the nucleolar constituents. It also suggested that forces of aggregation were preventing the separation of the lighter membranes from the heavier nonmembranous material. A method was therefore sought for better separating the two, and thus increasing the yield of purified nuclear membranes in the 1.6I fraction.

Effect of Removal of NMF from 0.5 M MgCl₂, Sucrose Buffer A—A possible insight into the nature of this aggregation effect was obtained by observing what happened to NMF after removal from 0.5 M MgCl₂ (see "Materials and Methods"). While in the high salt only about 12% of the protein sedimented at low speed (1000 × g, 10 min) (see Table I). When NMF was transferred to sucrose Buffer A, however, 6 times as much protein sedimented at 1000 × g. It thus appeared that the high concentration of MgCl₂, which was effective in removing residual DNA and nucleoplasm from the membrane fraction (2), also prevented the membrane from aggregating with the remaining nonmembranous material. Once taken out of high salt, aggregation rapidly took place between the two. This aggregation appeared, to a large extent, to be irreversible since resuspension of NMF in 0.5 M MgCl₂ sucrose Buffer A resulted in only a slight decrease in the sedimentation at 1000 × g (Table I).

Fractionation of NMF on 0.5 M MgCl₂, Sucrose Buffer A Gradients—Since high MgCl₂ concentration appeared to be effective in preventing the aggregation of nuclear membranes with nucleolar components, the NMF was fractionated on sucrose gradients without removal from 0.5 M MgCl₂. The procedure involved direct layering of the DNase-treated nuclei resuspended in 0.5 M MgCl₂ sucrose Buffer A on sucrose Buffer A gradients similarly made 0.5 M in MgCl₂ (see Fig. 3). The fractions were collected, washed once in 0.5 M MgCl₂ buffer, centrifuged at 104,000 × g for 30 min, washed once in sucrose Buffer A, and finally suspended in a small amount of sucrose Buffer A.

Since no change in distribution was detected in high salt sucrose gradient runs varying from 30 min to 16 hours, 30 min was selected for routine preparation. Fig. 6 indicates the average protein distribution. The majority of protein (62%) banded at 1.6I (ρ = 1.05 to 1.23). Only 26% and 12% of the protein banded at 2.2I (ρ = 1.23 to 1.30) and 2.2P, (ρ ≥ 1.30), respectively.

Electron microscopy revealed the membranous nature of the 1.6I fraction in sectioned view (Fig. 7). In contrast, the 2.2P consisted of nucleolar material of the granular-fibrillar type as well as more condensed nucleoli which displayed the characteristic lacunae (Fig. 8). Nuclear pore complexes, apparently dissociated from the nuclear membrane and occasional mitochondria were also present. Surface view of the fractions via negative staining with phosphotungstic acid confirmed these observations. The presence of nuclear pore complexes in the membranes of the 1.6I fraction identified it as nuclear membrane (see Fig. 9). Fig. 10 shows the negatively stained appearance of fragments of the granular-fibrillar nucleolar material. Very little membranous structure was detectable. The 2.2I fraction as observed in negative staining consisted of some nuclear membrane and larger amounts of nucleolar constituents.

Chemical Composition of High Salt Sucrose Gradient Fractions—Table II presents a compositional profile of the high salt sucrose gradient-fractionated NMF. The increased phospholipid content in 1.6I of 22%, as compared to 16% for NMF was indicative of the increased membrane content of 1.6I. The decrease in phospholipid in 2.2I and 2.2P (9.7% and 9.8%, respectively) relative to NMF conversely pointed to the enrichment of nonmembranous material in these fractions. The relative decrease in RNA content in 1.6I (5.8%), and progressive increases in 2.2I (8.0%) and 2.2P (11.4%) in comparison to 7.2% for NMF, indicated the high RNA content of at least some of the nonmembranous material. The presence of nucleolar constituents in 2.2P was consistent with the higher RNA content of this fraction.

**Table I**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Protein recovered in pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMF in 0.5 M MgCl₂ sucrose Buffer A (1000 × g, 10 min)</td>
<td>12.3%*</td>
</tr>
<tr>
<td>NMF in sucrose Buffer A (1000 × g, 10 min)</td>
<td>62.8%</td>
</tr>
<tr>
<td>NMF in sucrose Buffer A put in 0.5 M MgCl₂ sucrose Buffer A for periods up to 20 hours (1000 × g, 10 min)</td>
<td>57.5%</td>
</tr>
</tbody>
</table>

* The values represent an average of three determinations. The values in parentheses give the range.
FIG. 7. Thin section of the 1.61 fraction. The membranous nature of the fraction is clearly seen. × 30,000.

FIG. 8. Thin section of the 2.34 fraction. The structures observed in this fraction contrast sharply with those of the 1.61 fraction. Both condensed and granular-fibrillar forms of nucleolar material are present. Intact mitochondria and nuclear pore complexes are also observed. NE, condensed form of nucleolus; NEM, granular-fibrillar nucleolar material; M, mitochondria; NP, nuclear pore complexes. × 30,000.

FIGURE LEGENDS 9 & 10 on page 5555.
The DNA content of all the fractions was low (1.1 to 1.5%) but reproducible.

Concentration of Enzyme Activities in NMF—As described under "Materials and Methods," marker enzymes were used to evaluate possible mitochondrial and microsomal contamination in the nuclear fractions. We have previously reported that in nuclei most of the activities for certain endoplasmic reticulum-type enzymes, glucose 6-phosphatase, NADH-cytochrome c reductase, and Mg++-stimulated ATPase (oligomycin insensitive), as well as for the characteristically mitochondrial activity, cytochrome c oxidase, and a NADH oxidase activity, could not be attributed to microsomal and mitochondrial contaminations (1). Analysis of NMF gave similar results. Eighty to 90% of the activity for the above enzymes was not due to contamination. Moreover, the enzyme activities were highly concentrated in NMF. As demonstrated in Table III, the specific activities in NMF ranged from 4.0 to 7.9 times those in the nuclear fractions. We have previously reported that in purified nuclear membranes (1.61) as compared to 2.21 and 2.2P, the nucleoli-rich fraction, progressively decreased. This is presented in Table V where the activities in the gradient fractions are compared to those in NMF. The analysis indicated a 1.2 to 1.8-fold increase in specific activity in purified nuclear membranes for the characteristically ER enzymes glucose 6-phosphatase, NADH for reductase, NADH-cytochrome c reductase and Mg++-stimulated ATPase. In addition, cytochrome c oxidase and NADH oxidase activities were 1.9 times that of NMF. Decreases in these activities ranging from 0.32 to 0.61 and from 0.15 to 0.41 that of NMF were found in 2.21 and 2.2P, respectively. Moreover, between 54 to 90% of the total activity for the various enzymes was in the purified nuclear membrane fraction (Fig. 11). The 2.2P contained 7 to 15% of the total activity, while 2.2P had only 1 to 4%. Finally, the high salt sucrose gradients did not drastically affect enzymatic activities. On the contrary, the total activities were completely recovered with respect to NMF for all the enzymes with the exception of 71% for NADH-cytochrome c reductase and 87% for glucose 6-phosphatase (Table VI).

Since the enzyme activities are concentrated in terms of both specific and total activities in the purified nuclear membranes, their association with nuclear membranes is clearly indicated. The smaller amounts of activities in 2.21 can be ascribed to the nuclear membranes found in this fraction. The only trace amounts of activities in the nucleoli-rich 2.2P fraction suggest that nucleoli are devoid of the enzymes studied.

Enzymatic, Ultrastructural, and Chemical Analysis of Mitochondrial Contamination in Purified Nuclear Membranes—Since cytochrome c oxidase is an enzyme characteristic of inner mitochondrial membrane, detection of this membrane in the purified nuclear membranes was attempted by a number of parameters. Use of the marker enzyme approach indicated that mitochondrial contamination was at most very slight and contributed only small amounts to the cytochrome c oxidase activity measured in purified nuclear membranes (Table IV). In addition, the evaluated contamination was approximately the same in the three sucrose gradient fractions (3.6 to 3.8% mitochondrial protein), even though the cytochrome oxidase activity was more than 4 times higher in purified nuclear membranes (1.61) as compared to 2.21 and 2.2P. Ultrastructural observations supported these findings. No mitochondria were observed in purified nuclear membranes (see Fig. 7). In contrast, occasional mitochondria were visible in the nucleoli-rich 2.2P fraction (see Fig. 8).

Although relatively intact mitochondria are easily recognized in thin section, inner mitochondrial membranes are more difficult to identify in this manner. Inner mitochondrial membranes negatively stained, however, are characterized by 90 A particles which project from the surface of the membranes (37, 38). The membranes in the 1.61 fraction were identified as nuclear membranes by the presence of the nuclear pore complexes (39, 40). The DNA content of all the fractions was low (1.1 to 1.5%) but reproducible.

The DNA content of all the fractions was low (1.1 to 1.5%) but reproducible.

Concentration of Enzyme Activities in NMF—As described under "Materials and Methods," marker enzymes were used to evaluate possible mitochondrial and microsomal contamination in the nuclear fractions. We have previously reported that in nuclei most of the activities for certain endoplasmic reticulum-type enzymes, glucose 6-phosphatase, NADH-cytochrome c reductase, and Mg++-stimulated ATPase (oligomycin insensitive), as well as for the characteristically mitochondrial activity, cytochrome c oxidase, and a NADH oxidase activity, could not be attributed to microsomal and mitochondrial contaminations (1). Analysis of NMF gave similar results. Eighty to 90% of the activity for the above enzymes was not due to contamination. Moreover, the enzyme activities were highly concentrated in NMF. As demonstrated in Table III, the specific activities in NMF ranged from 4.0 to 7.9 times those in the nuclear fraction. Since Rees et al. (3) and Penniall and co-workers (4, 5) have both suggested the localization of nuclear electron transport activities in so-called nuclear fractions, it could not be unambiguously stated that all the enzyme activities in NMF were located on the nuclear membrane. The separation of nuclear membranes from the nucleolar constituents provides a means of resolving this question of enzyme localization.

Enzymatic Analysis of High Salt Sucrose Gradient Fractions—Table IV presents a enzyme profile of the high salt sucrose gradient fractions. The 1.61 fraction or purified nuclear membranes had the highest activities for all the nuclear enzymes studied. In contrast, the activities in 2.21 (containing both nuclear membranes and nucleolar components) and in 2.2P, the nucleoli-rich fraction, progressively decreased. This is presented in Table V where the activities in the gradient fractions are compared to those in NMF. The analysis indicated a 1.2 to 1.8-fold increase in specific activity in purified nuclear membranes for the characteristically ER enzymes glucose 6-phosphatase, NADH for reductase, NADH-cytochrome c reductase and Mg++-stimulated ATPase. In addition, cytochrome c oxidase and NADH oxidase activities were 1.9 times that of NMF. Decreases in these activities ranging from 0.32 to 0.61 and from 0.15 to 0.41 that of NMF were found in 2.21 and 2.2P, respectively. Moreover, between 54 to 90% of the total activity for the various enzymes was in the purified nuclear membrane fraction (Fig. 11). The 2.2P contained 7 to 15% of the total activity, while 2.2P had only 1 to 4%. Finally, the high salt sucrose gradients did not drastically affect enzymatic activities. On the contrary, the total activities were completely recovered with respect to NMF for all the enzymes with the exception of 71% for NADH-cytochrome c reductase and 87% for glucose 6-phosphatase (Table VI).

Since the enzyme activities are concentrated in terms of both specific and total activities in the purified nuclear membranes, their association with nuclear membranes is clearly indicated. The smaller amounts of activities in 2.21 can be ascribed to the nuclear membranes found in this fraction. The only trace amounts of activities in the nucleoli-rich 2.2P fraction suggest that nucleoli are devoid of the enzymes studied.

Enzymatic, Ultrastructural, and Chemical Analysis of Mitochondrial Contamination in Purified Nuclear Membranes—Since cytochrome c oxidase is an enzyme characteristic of inner mitochondrial membrane, detection of this membrane in the purified nuclear membranes was attempted by a number of parameters. Use of the marker enzyme approach indicated that mitochondrial contamination was at most very slight and contributed only small amounts to the cytochrome c oxidase activity measured in purified nuclear membranes (see Table IV). In addition, the evaluated contamination was approximately the same in the three sucrose gradient fractions (3.6 to 3.8% mitochondrial protein), even though the cytochrome oxidase activity was more than 4 times higher in purified nuclear membranes (1.61) as compared to 2.21 and 2.2P. Ultrastructural observations supported these findings. No mitochondria were observed in purified nuclear membranes (see Fig. 7). In contrast, occasional mitochondria were visible in the nucleoli-rich 2.2P fraction (see Fig. 8).

Although relatively intact mitochondria are easily recognized in thin section, inner mitochondrial membranes are more difficult to identify in this manner. Inner mitochondrial membranes negatively stained, however, are characterized by 90 A particles which project from the surface of the membranes (37, 38). The membranes in the 1.61 fraction were identified as nuclear membranes by the presence of the nuclear pore complexes (39, 40).
TABLE IV

**Enzyme profile of high salt sucrose gradient fractionated NMF**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Mitochondrial activity</th>
<th>Microsomal activity</th>
<th>LM Activity</th>
<th>LM Activity</th>
<th>LM Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mitochondrial marker</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate dehydrogenase (μmoles 2,6-dichlorophenolindophenol/min/mg protein)</td>
<td>(5) 0.009 (3.4%)</td>
<td>(11) 0.0034 (3.7%)</td>
<td>(12) 0.0039 (3.8%)</td>
<td>(11) 0.0038 (3.6%)</td>
<td></td>
</tr>
<tr>
<td><strong>Microsomal marker</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (μmoles cytochrome c/min/mg protein)</td>
<td>(5) 0.080 (5.5%)</td>
<td>(11) 0.0044 (6.5%)</td>
<td>(10) 0.0052 (6.1%)</td>
<td>(9) 0.0025 (3.1%)</td>
<td></td>
</tr>
<tr>
<td><strong>Enzymes endogenous to nuclei</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH-ferricyanide reductase (μmoles K₃Fe(CN)₄/min/mg protein)</td>
<td>(4) 3.48 6.03 1.62 2.59</td>
<td>(8) 1.19 0.42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH-cytochrome c reductase (μmoles cytochrome c/min/mg protein)</td>
<td>(3) 2.00 0.71 0.84</td>
<td>(6) 0.339 0.107</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH oxidase (μmoles O₂/min/mg protein)</td>
<td>(5) 0.272 (11) 0.134 0.251</td>
<td>(11) 0.080 0.021</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c oxidase (μmoles O₂/min/mg protein)</td>
<td>(5) 0.819 (11) 0.115 0.213</td>
<td>(10) 0.051 0.047</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose 6-phosphatase (μmoles Pi/10 min/mg protein)</td>
<td>(4) 2.45 0.805 1.20 0.403</td>
<td>(8) 0.157</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg⁺⁺-stimulated ATPase (μmoles Pi/10 min/mg protein)</td>
<td>(8) 1.72 0.947 1.14 0.206</td>
<td>(8) 0.208</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The numbers in parentheses above the specific activities represent the number of determinations. The percentages below the specific activities are the percentage of mitochondrial or microsomal contaminating protein.*

**TABLE V**

**Comparison of enzyme-specific activities in high salt sucrose gradient fractions to NMF**

Specific activities were taken from Table IV.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Ratio of 1.6 to NMF</th>
<th>Ratio of 2.3 to NMF</th>
<th>Ratio of 2.5 to NMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate dehydrogenase</td>
<td>1.2 1.1 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH-tetrahydride reductase</td>
<td>1.6 0.61 0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH-cytochrome c reductase</td>
<td>1.2 0.48 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>1.9 0.60 0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>1.9 0.44 0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose 6-phosphatase</td>
<td>1.5 0.50 0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg⁺⁺-stimulated ATPase</td>
<td>1.8 0.32 0.32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

and were devoid of membranes with projecting particles (see Fig. 9).

Moreover, chemical evaluation of typical inner mitochondrial membrane components correlated with the enzymatic and ultrastructural results.

Coenzyme Q (ubiquinone) is a characteristic component of the inner mitochondrial membrane (41). Table VII demonstrates its presence in mitochondria in the amount of 2.06 nmol of coenzyme Q per mg of protein, which agrees with literature values for rat liver mitochondria (41-43). In contrast, no coenzyme Q was detected in nuclear membrane lipids by spectrophotometric assay of lipid extracts or by visual inspection of leucomyelene blue-treated thin layer plates. In order to estimate the minimum amount of coenzyme Q present in purified nuclear membranes, the limit of detection of coenzyme Q on thin layer plates was determined by estimating the point at which a blue spot, produced by spraying the plate with reduced methylene blue, was no longer visible. Up to 0.25 nmol of coenzyme Q can be detected in this manner. With this as an upper limit, the maximum coenzyme Q content in nuclear membranes was 0.054 nmol per mg of protein, representing a maximum of 2.5% mitochondria contamination.

Mitochondria contained 0.414 nmol of acid-extractable flavin and 0.266 nmol of acid-nonextractable, trypsin-releasable flavin on a milligrams of protein basis (Table VII). These values are similar to those reported for rat liver mitochondria (44). Acid-extractable flavin (0.399 nmol) was detected in purified nuclear membranes, but no acid-nonextractable, trypsin-releasable flavin was present (Table VII). Since acid-nonextractable, trypsin-releasable flavin is believed to be associated with the succinate dehydrogenase flavoprotein (45, 46), which is a component of the inner mitochondrial membrane, the absence of trypsin releasable flavin in nuclear membranes is consistent with the low levels of succinate dehydrogenase estimated enzymatically (Table IV). It is therefore concluded that the purified nuclear membrane preparations are devoid of both intact mitochondria and inner mitochondrial membranes.

**Analysis of Microsomal and Outer Mitochondrial Membranes in**
TABLE VI
Recovery of total activity in high salt sucrose gradient fractionated nuclear membranes with respect to NMF

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>NMF</th>
<th>1.6I</th>
<th>2.2I</th>
<th>2.2P</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-ferricyanide reductase</td>
<td>101</td>
<td>89 (81%)</td>
<td>14.9</td>
<td>1.1</td>
<td>97</td>
</tr>
<tr>
<td>NADH-cytochrome c reductase</td>
<td>44.4</td>
<td>26.6 (60%)</td>
<td>4.2</td>
<td>0.6</td>
<td>71</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>8.4</td>
<td>8.0 (99%)</td>
<td>1.0</td>
<td>0.1</td>
<td>108</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>7.2</td>
<td>6.7 (93%)</td>
<td>0.6</td>
<td>0.3</td>
<td>106</td>
</tr>
<tr>
<td>Glucose 6-phosphatase</td>
<td>50.3</td>
<td>38.0 (76%)</td>
<td>5.0</td>
<td>0.9</td>
<td>87</td>
</tr>
<tr>
<td>Mg2+-ATPase</td>
<td>40.4</td>
<td>36.1 (89%)</td>
<td>2.6</td>
<td>1.2</td>
<td>99</td>
</tr>
</tbody>
</table>

*Total activity was calculated by multiplying the average protein content by the corrected specific activity given in Table IV. The average proteins based on an average of 62.5 mg of NMF for five separate preparations were 31.7 mg for 1.6I, 12.5 mg for 2.2I, and 5.8 mg for 2.2P.

Purified Nuclear Membranes—Microsomal vesicles, which are derived from the endoplasmic reticulum, are identified in negative staining by a phosphotungstic acid-repellent layer surrounding the membrane vesicles (47), and are further distinguished from nuclear membranes by the absence of pore complexes. Microsomal-like vesicles were only occasionally recognized in the purified nuclear membrane fraction (see Fig. 9). The small amount of microsomal membrane observed ultrastructurally was paralleled by trace amounts of the ER enzyme activity NADPH-cytochrome c reductase. With this activity as a marker, only 10 to 15% of NADH-cytochrome c reductase, glucose 6-phosphatase, and Mg2+-stimulated ATPase activities in the purified nuclear membranes could be accounted for by microsomal contamination (see Table IV). If a part or all of the NADPH-cytochrome c reductase is actually endogenous to nuclear membranes, as has been suggested elsewhere (48-50), then the small contribution of microsomal contamination to the above ER type enzymes in nuclear membranes is reduced accordingly.

Monoamine oxidase activity was not detected in purified nuclear membranes, indicating the absence of outer mitochondrial membranes (24).

Cytochrome b5—Fig. 12 demonstrates that the difference spectra of nuclear membranes and microsomes both contain absorption peaks characteristic of cytochrome b5 (556, 526, and 426 nm). The b5 content of nuclear membranes, corrected for contamination, was 0.322 nmoles per mg of protein compared to 1.02 nmoles per mg of protein for microsomes (Table VIII).

Cytochrome P-450—As shown in Fig. 13, the reduced carbon monoxide versus reduced difference spectrum of nuclear mem-
branes reveals an absorption peak at 450 nm. This peak, however, is relatively small compared to the peak at 450 nm displayed by microsomes (Fig. 13). Microsomes also contained a small amount of P-420 as judged by the peak at 425 nm. No P-420 was detected in nuclear membranes. Further proof that the absorption peaks at 450 nm actually correspond by cytochrome P-450 was obtained by adding deoxycholate to both cuvettes. This shifts the 450-nm peak to a 425-nm peak characteristic of P-420 which is a solubilized form of P-450 (see references 20 and 51). The P-450 content of nuclear membranes and microsomes was 0.003 nmole and 1.24 nmol per mg of protein (Table VIII). After subtracting possible microsomal contamination as evaluated via NADPH-cytochrome c reductase activity, the P-450 content of nuclear membrane reduces to zero.

Cytochrome a, a₃—Mitochondria cytochrome c oxidase contains the heme components cytochrome a₁, a₂. The a-band of cytochrome a₁, a₂ at 604 to 605 nm and the Soret band at 445 nm are characteristic of P-420 which is a solubilized form of P-450 (see ref-erences 20 and 51). The P-450 content of nuclear membranes and microsomes was 0.003 nmole and 1.24 nmol per mg of protein (Table VIII). After subtracting possible microsomal contamination as evaluated via NADPH-cytochrome c reductase activity, the P-450 content of nuclear membrane reduces to zero.

Cytochrome a, a₃—Mitochondria cytochrome c oxidase contains the heme components cytochrome a₁, a₂. The a-band of cytochrome a₁, a₂ at 604 to 605 nm and the Soret band at 445 nm are characteristic of P-420 which is a solubilized form of P-450 (see references 20 and 51). The P-450 content of nuclear membranes and microsomes was 0.003 nmole and 1.24 nmol per mg of protein (Table VIII). After subtracting possible microsomal contamination as evaluated via NADPH-cytochrome c reductase activity, the P-450 content of nuclear membrane reduces to zero.

Cytochrome a, a₃—Mitochondria cytochrome c oxidase contains the heme components cytochrome a₁, a₂. The a-band of cytochrome a₁, a₂ at 604 to 605 nm and the Soret band at 445 nm are characteristic of P-420 which is a solubilized form of P-450 (see references 20 and 51). The P-450 content of nuclear membranes and microsomes was 0.003 nmole and 1.24 nmol per mg of protein (Table VIII). After subtracting possible microsomal contamination as evaluated via NADPH-cytochrome c reductase activity, the P-450 content of nuclear membrane reduces to zero.

**Table VIII**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Mitochondria</th>
<th>Microsomal membranes</th>
<th>Nuclear membranes</th>
<th>Nuclear membranes corrected for contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate dehydrogenase*</td>
<td>0.009</td>
<td>0.0014</td>
<td>0.0037</td>
<td></td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase*</td>
<td>0.080</td>
<td>0.0052</td>
<td>0.0032</td>
<td></td>
</tr>
<tr>
<td>Cytochrome b₅*</td>
<td>1.02</td>
<td>0.398</td>
<td>0.332</td>
<td></td>
</tr>
<tr>
<td>Cytochrome a₁, a₂*</td>
<td>1.24</td>
<td>0.055</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>Cytochrome oxidase*</td>
<td>0.152</td>
<td>0.051</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>Cytochrome oxidase*</td>
<td>0.012</td>
<td>0.284</td>
<td>0.254</td>
<td></td>
</tr>
<tr>
<td>Ratio of cytochrome a₁, a₂ to</td>
<td>0.186</td>
<td>0.177</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Micromole of 2,6-dichlorophenolindophenol reduced per min per mg of protein.

**Fig. 13.** Reduced carbon monoxide versus reduced difference spectra. A, microsomes (0.43 mg of protein per ml); B, nuclear membranes (2.0 mg of protein per ml).

**Fig. 14.** Reduced versus oxidized difference spectra. Turbidity was reduced by the addition of deoxycholate as described under “Materials and Methods.” A, mitochondria (9.0 mg of protein per ml); B, nuclear membranes (9.0 mg of protein per ml).
relative to microsomes is presented in Table IX. NADH-cytochrome c and NADH-ferricyanide reductases were present in nuclear membranes at a level of 42 to 43% of microsomes. Similarly, glucose 6-phosphatase is 49% of microsomes, while Mg++-stimulated ATPase and cytochrome b$_5$ were 66% and 33% of the microsomal values. Nuclear membranes therefore have a very strong microsomal character enzymatically.

**DISCUSSION**

*Binding of Exogenous Material to Nuclear Membranes*—The major difficulty encountered in separating nuclear membranes from contaminating nucleolar constituents was an apparent aggregation which prevented their separation on conventional sucrose gradients. By maintaining NMF in a high salt environment (0.5 M MgCl$_2$), aggregation was prevented, and the membranes were successfully separated from the nucleolar components.

The finding that two components with a large density difference bind together and sediment at a "hybrid density" suggests that banding at a particular density in sucrose gradients does not necessarily imply homogeneity of the fraction. The possibility of binding of two or more components of different densities to form artifacts should be carefully evaluated.

The somewhat divergent data concerning the composition of rat liver nuclear membranes (see References 48–50 and 56) may, to some extent at least, be explained by the binding of exogenous material to nuclear membranes. Alternatively, differences in chemical composition may reflect variations in the relative amounts of outer and inner nuclear membranes, as well as the relative amounts of nuclear pore material (40).

*Localization of Enzyme Activities in Nuclear Membranes*—Analysis of the high salt sucrose gradient fractions demonstrated the concentration of certain enzymes in the purified nuclear membrane fraction (1.61) in terms of both specific and total activities. An intermediate fraction (2.21), consisting of both nuclear membranes and nucleolar material, contained much less of the activities, while the nucleoli-rich fraction (2.2P) was devoid of the enzymes studied. It is therefore concluded that glucose 6-phosphatase, Mg++-stimulated ATPase, NADH-cytochrome c reductase, cytochrome c oxidase, and NADH oxidase activities are endogenous to nuclear membranes and absent from nucleoli. Since electron microscopic observations were not emphasized, the earlier reports of electron transport components in so-called nucleolar preparations (3–5) could very well have been due to nuclear membranes contaminating the preparations. The strong aggregation between nuclear membranes and nucleolar components found in this study supports this view.

*Enzyme and Cytochrome Profile of Nuclear Membranes*—The trace amount of succinate dehydrogenase activity in nuclear membranes corresponded to the small level of mitochondrial contamination as evaluated ultrastructurally and by the absence of the mitochondrial inner membrane components cytochrome b$_5$ (57) and coenzyme Q (41). The validity of the enzymatic evaluation was supported by the inability to detect acid-nonextractable, trypsin-releasable flavin in nuclear membranes. Therefore the trace activity for succinate dehydrogenase actually corresponds to only trace quantities of the flavoprotein succinate dehydrogenase, rather than to an inactivation of the dehydrogenase. Succinate dehydrogenase and succinate oxidase were also not detected in rat liver nuclear membranes by Zbarsky et al. (58) and Kuzmina et al. (59), respectively.

There is considerable variation in reported values for NADPH-cytochrome c reductase activity and cytochrome P-450 content in mammalian nuclear membrane preparations. Franke et al. (49) reported low values for cytochrome P-450 in pig and rat liver nuclei membranes similar to the value reported here for bovine liver, but Ichikawa and Mason (60), with the use of an isolation procedure similar to Franke et al. (49) found a 10-fold higher cytochrome P-450 content in rabbit liver nuclear membranes. Furthermore, although Kasper (61) reported four times as much cytochrome P-450 as in bovine liver, he was unable to detect the cytochrome c in 7 out of 10 rat liver preparations. Zbarsky et al. (48) reported NADPH-cytochrome c reductase activity in rat liver similar to bovine liver, but Kasper (61), Franke et al. (49) and Ichikawa and Mason (60) all demonstrated much higher specific activities in rat, pig, and rabbit membrane preparations.

The specific activity of NADH-cytochrome c reductase in bovine liver was slightly higher than that found by Kasper (61) for rat liver and much higher than the specific activities reported by both Franke et al. (49) and Zbarsky et al. (48). The low NADH-cytochrome c reductase specific activity demonstrated by Franke et al. was paralleled by a relatively low level (0.034 mmole per mg of protein) of cytochrome b$_5$ (49), whereas both Kasper (61) and Ichikawa and Mason (60) reported values for cytochrome b$_5$ similar to bovine liver.

Both these studies and those of Kashnig and Kasper (50) demonstrated glucose 6-phosphatase in relatively high specific activities while Franke et al. (49) and Zbarsky et al. (48) detected only trace amounts of glucose 6-phosphatase. Recently, however, E.-D. Jarasch of Franke’s group has detected much higher levels of glucose 6-phosphatase in their rat liver nuclear membrane preparations (0.25 mmole of P$_1$ per 10 min per mg of protein). Thus current biochemical evidence favors the presence of glucose 6-phosphatase in nuclear membranes, a conclusion which is further supported by histochemical analysis (62–66).

Data concerning ATPase activity are much more consistent. All the groups have found Mg++-stimulated ATPase in significant amounts (48–50); cytochrome oxidase activity in bovine liver nuclear membranes was found at a level ranging from 20 to 30% of control mitochondria. Zbarsky et al. (48) also reported cytochrome oxidase activity in rat liver nuclear membranes. Confirming these studies, E.-D. Jarasch has calculated cytochrome oxidase activity in rat liver nuclear membranes (prepared according to Franke et al. (49)) comparable to bovine liver (0.180 mmole of O$_2$ per min per mg of protein). It is therefore concluded

---

**Table IX**

Relative percentage of endoplasmic reticulum-type enzymes in nuclear membranes compared to microsomes

<table>
<thead>
<tr>
<th>Assay</th>
<th>Percentage of activity in nuclear membranes relative to activity in microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-ferricyanide reductase</td>
<td>43</td>
</tr>
<tr>
<td>NADH-cytochrome c reductase</td>
<td>42</td>
</tr>
<tr>
<td>Glucose 6-phosphatase</td>
<td>49</td>
</tr>
<tr>
<td>Mg++-stimulated ATPase</td>
<td>66</td>
</tr>
<tr>
<td>Cytochrome b$_5$</td>
<td>33</td>
</tr>
<tr>
<td>Cytochrome P-450</td>
<td>4.4</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*a* Actual activities are obtained from Table IV.

---

2 E.-D. Jarasch, personal communication.
that cytochrome c oxidase is a characteristic enzyme of liver nuclear membrane preparations.

Comparison of Enzyme Activities and Cytochromes in Nuclear and Microsomal Membrane—As demonstrated in Table IX, nuclear membranes resemble microsomes with respect to certain characteristic ER-type enzymes. Kashin and Kasper (50) have reported similar results and postulated that the enzymes would be located on only one of the membranes, presumably the outer nuclear membrane. If this were the case, and assuming that approximately 50% of the preparation was outer membrane, then specific activities for the NADH reductases glucose 6-phosphate, and Mg++-stimulated ATPase would be identical with those of rough and smooth microsomes. There is no a priori biochemical justification, however, for assuming that all these enzyme activities are located on the outer nuclear membrane. Indeed many combinations are possible.

Bovine liver nuclear membranes differed from microsomes by the relatively small amounts or absence of the NADPH electron transport components, NADPH-cytochrome c reductase, and cytochrome P-450. In addition, the nuclear membranes also contained the “nannomicosomal” enzyme cytochrome oxidase. Caution must be observed in interpreting this finding, however. If, for example, all the cytochrome oxidase was localized on the inner nuclear membrane, then the outer nuclear membrane may have solely ER-type enzymes. The inner nuclear membrane, in turn, may contain none of the ER-type enzymes, or alternatively, contain some and thus have partial ER character. Recently Ueda et al. (67) and Conover (68) have suggested that approximately 50% of the preparation was outer membrane, then specific activities for the NADH reductases, glucose 6-phosphate, and Mg++-stimulated ATPase would be identical with those of rough and smooth microsomes. There is no a priori biochemical justification, however, for assuming that all these enzyme activities are located on the outer nuclear membrane.

Acknowledgments—The authors are grateful to Mrs. Kathy J. Jewell, Mrs. Keri R. Safranski, and Mr. Steven A. Dodge for the preparation of subcellular fractions.

REFERENCES
13. Dische, Z. (1930) Mikrochemie 8, 4
29. Schneider, W. C. (1959) Advan. Enzymol. 21, 1
58. Zbarsky, I. B., Pokrovsky, A. A., Pevrchoski, K. A.,
5561


52. TRICE, L. W., AND BARRETT, R. J. (1961) J. Histochem. Cytochem. 9, 635

63. GOLDFISCHER, J., ESSNER, E., AND NOVIKOFF, A. B. (1964) J. Histochem. Cytochem. 12, 72


The Purification and Biochemical Characterization of Bovine Liver Nuclear Membranes
Ronald Berezney, Linda K. Macaulay and Frederick L. Crane


Access the most updated version of this article at http://www.jbc.org/content/247/17/5549

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/17/5549.full.html#ref-list-1