Biochemical Distinctions between the Nuclear and Microsomal Membranes from Rat Hepatocytes

THE EFFECT OF PHENOBARBITAL ADMINISTRATION*

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SUMMARY

The nuclear membrane from rat hepatocytes has been examined for components of the DPNH and TPNH electron transport chains as well as two drug-metabolizing enzymes, benzo[a]pyrene hydroxylase and an aminoozo dye N-demethylase, in control and phenobarbital-treated animals. The enzyme induction phenomenon characteristic of the microsomes was not observed with the nuclear membrane for any of the enzymes or pigments studied.

The nuclear electron transport enzyme DPNH-cytochrome c reductase and cytochrome b₅ were found to follow the same pattern as their microsomal counterparts. Thus, in each case, the reductase level was depressed in the treated animals while the cytochrome b₅ level was not significantly altered.

In control animals, the specific activity of TPNH-cytochrome c reductase in the nuclear membrane was approximately one-third that found for the same enzyme associated with the microsomes. The specific activity of this enzyme remained unchanged after phenobarbital was administered.

The occurrence of cytochrome P-450 in the nuclear membrane was variable. No cytochrome P-450 was demonstrable in membranes from phenobarbital-treated animals, but low levels were detected in three out of 10 individual control preparations. Measurable amounts of aryl hydroxylase and N-demethylase were found in the nuclear membrane which corresponded to 7 and 11% of the specific activity obtained for the same enzymes in the microsomal membrane, respectively. Failure of these enzymes to be induced suggests that they are not of microsomal origin.

Clearly, the differential response of the nuclear and microsomal membranes to phenobarbital indicates that quite distinct regulatory mechanisms must be operative for the two membranes with respect to the metabolic control of TPNH-cytochrome c reductase and the drug-metabolizing enzymes.

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Since the nucleus occupies a strategic position in the control and regulation of cell growth and differentiation, it is of importance to define in a precise manner the chemical and biochemical properties of the nuclear membrane. Questions regarding nuclear function frequently require a detailed knowledge of membrane structure and function. Studies from our laboratory (1, 2) and elsewhere (3-10) have dealt with the isolation, composition, morphology, and biochemistry of this unique bilayered structure. Morphologically, the outer leaflet of the nuclear membrane forms a continuum with the endoplasmic membranes (11), and it is of extreme interest to study the biochemical response of the nuclear membrane under various physiological conditions in an attempt better to define its functional role in the cell.

The main objective of this study was to examine further the biochemical interrelationships between the nuclear and microsomal membranes under conditions of enzyme induction. The response of various membrane-associated enzymes to phenobarbital administration is rather well characterized in the case of the microsomal system (12). For example, phenobarbital induces a spectrum of drug-metabolizing enzymes which require TPNH and molecular oxygen. Concomitant with this increase in drug-metabolizing capacity is an increase in the level of the components of the TPNH electron transport chain which includes TPNH-cytochrome c reductase (thought to be equivalent to cytochrome P-450 reductase) and cytochrome P-450 (13-15). This behavior is in contrast to the microsomal DPNH electron transport chain which is not induced by phenobarbital (14).

This communication describes the systematic examination of the nuclear membranes for the DPNH- and TPNH-dependent electron transport systems as well as for two representatives of the drug-metabolizing enzymes, N-demethylase and benzo[a]pyrene hydroxylase.

METHODS AND MATERIALS

Animals—Male Holtzman rats weighing 50 to 60 g were used in all studies. Induction of drug-metabolizing enzymes was accomplished by one daily intraperitoneal injection of 100 mg of phenobarbital per kg of body weight (14). Control animals received 0.9% NaCl by the same route. All animals were injected for 5 successive days in the mornings so as not to disturb feeding and sleeping cycles (16).

Preparation of Nuclear and Microsomal Membranes—Animals were fasted for a period of 20 hours and killed by decapitation. All subsequent operations were carried out at either ice bath temperature or 0°, and maximum values are given for the relative centrifugal force. The livers were homogenized in 2 vol...
umes of 0.25 M sucrose-buffer TKM (0.5 M Tris-HCl, pH 7.5, containing 0.025 M KCl and 0.005 M MgCl₂), filtered through four layers of cheesecloth, and centrifuged at 3,000 × g for 10 min in a Sorvall RC-3 centrifuge equipped with a HG-4 rotor. The crude nuclear pellet was resuspended in 3 to 4 volumes of 0.25 M sucrose-Buffer TKM, diluted with 2 volumes of 2.3 M sucrose-Buffer TKM, rehomogenized in a Potter-Elvehjem homogenizer, and underlayered with 2.3 M sucrose-Buffer TKM in a cellulose nitrate tube to fit the Spinco SW 25.2 rotor. Nuclei were sedimented through the high density sucrose by centrifuging at 106,000 × g for 65 min. The recovery of DNA in the washed nuclear pellet was in the range of 50 to 70% of the DNA present in the homogenate. Nuclear membrane comprising both the inner and outer leaflets was prepared as previously described (2).

The distribution of nuclear membrane between the density (d) 1.16 to 1.18 and d 1.18 to 1.20 g cc⁻¹ was approxi-mately 10:1 on a protein basis. Both fractions were combined for the purpose of this study. Routinely, 100 g of liver were processed per preparation and the average yield of nuclear mem-brane from control and phenobarbital-treated rats was 10 mg and 6 mg of membrane protein per 100 g of liver. The supernatant recovered from the 3,000 × g run was re-centrifuged at 14,000 × g for 10 min in order to remove nonmicromosomal contaminants. Crude microsomal membrane was obtained by cen-trifugation of the postmitochondrial supernatant at 106,000 × g for 60 min. The microsomal membrane was further purified by density gradient centrifugation on a discontinuous sucrose-citrate gradient by the method of Kashnig and Kasper (2). The above procedure permitted the isolation of nuclear and micro-somal membranes from the same liver homogenate.

Enzyme Assays—DPNH cytochrome c reductase was assayed by the method of Mackler and Green (17) with 0.50 ml of cock-tail. The final volume after the addition of enzyme and cofactor was 0.58 ml. For the determination of TPNH-cytochrome c reductase the same assay was used but 100 μg of TPNH were substituted for DPNH. All incubations were carried out at 30° in a thermostated cuvetto chamber of a Beckman DB-C spectrophotometer and the amount of cytochrome c reduced was cal-culated with the extinction coefficient at 550 μm of 18.5 cm⁻¹ mm⁻¹ (18). TPNH and DPNH were obtained as the tetra-sodium salts from Sigma.

N-Demethylase activity was determined by measuring the oxidative demethylation of 3-methyl-4-monomethylaminoazobenzene by a modification of the procedure of Mueller and Miller (19). The assay was conducted in 0.05 M Tris-HCl buffer, pH 7.5, containing 25 μg of 3-methyl-4-monomethylaminoazobenzene, 522 μg of MgCl₂, 2 mg of KCl, 500 μg of TPNH, and 0.5 mg to 2 mg of microsomal or nuclear membrane protein in a final vol-ume of 1 ml. The reaction was initiated by the addition of TPNH and the incubation was carried out aerobically with shak-ing at 37° for 30 min. A TPNH-regenerating system was not required. The reaction was stopped by the addition of 1.5 volumes of acetone and 1.5 volumes of benzene. After vigorous mixing, 1.0 ml of the organic phase was removed and the solvent was evaporated in a stream of nitrogen. The residue was redis-solved in 25 μl of methanol and a 5-μl aliquot was applied to an activated previously coated silica gel plate (Eastman, type 6061). The chromatogram was developed at room temperature for 4 hours with a methanol-hexane solvent (4:96). After removal of the solvent, the visualization of the dyes was aided by brief exposure of the thin layer chromatographic plate to HCl vapor.

3-Methyl-4-monomethylaminoazobenzene and the major product 3-methyl-4-aminooazobenzene had Rₚ values of 0.36 and 0.14, respectively. The area of the chromatogram containing each of these compounds was cut out and transferred to individual conical centrifuge tubes. Methanol (0.06 ml) was added to each tube and the silica gel was dislodged from the Mylar backing by vortex mixing. This step also extracted the dyes from the silica gel. An equal volume of 7 N HCl was added to the methanol extract with mixing, the Mylar backing was removed, and the contents of the tube were centrifuged to obtain an optically clear solution. Concentrations were determined by comparison to a standard curve after the optical density was measured at 505 μm with a Beckman DU-2 spectrophotometer.

Benzofalpyrene hydroxylase was assayed by the procedure of Nebert and Gelboin (20). Pure samples of benzo[a]pyrene and the 8-hydroxy derivative were kindly provided by Dr. J. A. Miller. Fluorescence was determined on a G. K. Turner Fluoro-meter equipped with filters 7-80 and 58 which transmit light maximally at 396 μm and 522 μm, respectively.

Cytochrome b₅ was determined from the difference spectrum between the oxidized and reduced form. DPNH was used as the reducing agent at a final concentration of 0.2 μg per ml. All spectra were measured in 0.23 M Tris buffer, pH 7.5, containing 50% glycerol with a Cary model 15 spectrophotometer. An extinction coefficient of 163 cm⁻¹ mm⁻¹ was used for the absorption difference between 424 μm and 409 μm (21). Cytochrome P-450 was determined on the same sample. After reduction of the suspensions in the reference and sample cuvettes with sodium dithionite, carbon monoxide was gently passed through the contents of the sample cuvette for 20 to 30 sec prior to re-cording the difference spectrum. An extinction difference coeffi-cient of 91 cm⁻¹ mm⁻¹ between 450 μm and 490 μm was used to calculate the amount of CO-heme complex (22).

Protein was determined by the Folin procedure (23) with four times crystallized ovalbumin as the standard.

RESULTS AND DISCUSSION

Nuclear and microsomal membranes were prepared from the same animals so that precise correlations of enzymic activities could be made. As isolated, the nuclear membrane is a complex comprising both the inner and outer leaflets. In control animals, DPNH-cytochrome c reductase was found in the nuclear mem-brane at a level approximately 56% that found for the micro-somal membrane (Table I); this finding is consistent with earlier results from our laboratory which also showed the rotenone insensitivity of nuclear-DPNH-cytochrome c reductase (2). Berecky, Funk, and Crane (10) have obtained a similar result (55%) with bovine liver nuclear membrane while Zbarsky et al. (6) and more recently Franke et al. (7) have reported somewhat lower values of 21% and 30%, respectively, for nuclear mem-brane from rat liver. Nuclear and microsomal membranes isolated from induced animals exhibited a 27% and 34% decrease in the specific activity of DPNH cytochrome c reductase, respec-tively. Orenius, Ericsson, and Ernster (14), by measuring DPNH oxidation, reported a 56% drop in specific activity in rat liver microsomes after treatment with phenobarbital for 5 days. Arias, Doyle, and Schimke (24) have demonstrated that phenobarbital actually decreased the rate of synthesis of some mem-brane proteins while increasing total microsomal protein synthesis about 2-fold. Thus, the decrease in reductase activity may be due to a combination of factors such as reduced synthesis, in-
The values presented in this table represent averages with standard deviations for control and phenobarbital-treated groups of animals. The number of animals in the control and treated groups was approximately 60 and 40, respectively. Each value represents the average of a minimum of four groups of animals.

### Table I

| Membrane fraction | DPNH-cytochrome c reductase | TPNH-cytochrome c reductase | Cytochrome b$_5$ | Cytochrome P-450 | N-Demethylase | Aryl hydroxylase |
|-------------------|-----------------------------|-----------------------------|------------------|-----------------|---------------|-----------------|
| Nuclear membrane  |                             |                             |                  |                 |               |                 |
| Control           | 0.552 ± 0.080               | 0.104 ± 0.015               | 0.181 ± 0.020    | 0.0 ± 0.22      | 2.6 ± 0.4     | 259 ± 27        |
| Induced           | 0.401 ± 0.062               | 0.092 ± 0.010               | 0.210 ± 0.035    | None detected   | 3.1 ± 0.8     | 208 ± 30        |
| Microsomal membrane |                           |                             |                  |                 |               |                 |
| Control           | 0.981 ± 0.150               | 0.322 ± 0.041               | 0.402 ± 0.095    | 0.62 ± 0.07     | 24.1 ± 3.0    | 3,640 ± 525     |
| Induced           | 0.653 ± 0.098               | 0.630 ± 0.066               | 0.408 ± 0.110    | 1.57 ± 0.31     | 51.6 ± 6.2    | 11,860 ± 1,300  |

* Micromoles of cytochrome c reduced per min per mg of protein.
* Millimicromoles per mg of protein.
* Millimicromoles of 3-methyl-4-aminoazobenzene formed per 30 min per mg of protein.
* Micromicromoles of hydroxylated benzo[a]pyrene formed per 30 min per mg of protein.

The pigment, cytochrome $b_5$, a component of the DPNH electron transfer chain was found in nuclear membrane from control animals at 37% of the level occurring in the microsomes (Table I). The cytochrome $b_5$ content of 0.193 mmole per mg of nuclear membrane protein is greater than 5 times the content reported by Franke et al. (7) for the same membrane. It is well known that the nucleus contains proteolytic enzymes (28), and it is possible that the prolonged incubation of a sonicated nuclear suspension (7) could have resulted in the release of some membrane proteins through the action of proteases. Cytochrome $b_5$ is released in good yield from the microsomal membrane by mild proteolytic digestion (25-28). Phenobarbital induction did not significantly alter the cytochrome $b_5$ content of the nuclear membrane and in this respect is quite similar to the pattern obtained for the microsomal membrane (Table I; Reference 14). Presumably, the induction process has little effect on the rate of cytochrome $b_5$ synthesis in microsomes (24). Furthermore, cytochrome $b_5$ degradation is completely prevented during phenobarbital administration (29). Our results would suggest that a similar situation may exist for the nuclear membrane.

TPNH-cytochrome c reductase was found to be an integral part of the nuclear membrane with a specific activity close to one-third of the microsomal membrane. This reductase is generally assumed to be identical with the enzyme that transfers reducing equivalents from TPNH to cytochrome P-450 (30). Other investigators have also reported the occurrence of this enzyme in rat liver nuclear membrane (6, 7). However, in the case of bovine liver nuclear membrane, TPNH-cytochrome c reductase appears to be absent (10). A most important distinction exists between the nuclear and microsomal membranes. The TPNH-cytochrome c reductase associated with the nuclear membrane is not inducible by phenobarbital while the level of the microsomal enzyme is increased 2- to 4-fold over control values (Table I; Reference 14). This finding clearly illustrates a marked difference in the effect of phenobarbital on two intracellular membranes.

Analysis of the nuclear membrane for cytochrome P-450 yielded variable results. Ten individual preparations from control animals were examined and negative results were obtained with seven of the samples. In three preparations, cytochrome P-450 was present at the level of 0.08, 0.16, and 0.22 mmole per mg of protein. These values correspond to 13, 26, and 35% of the value obtained for the microsomal membrane. All of the analyses were performed at a protein concentration in the range of 2 to 4.5 mg per ml. Thus, the level at which the analyses were carried out would have permitted the detection of cytochrome P-450 down to the 10% level in most cases. The reason for not obtaining uniform results in the determination of this pigment is unclear. No cytochrome P-420 was observed in any of the nuclear membrane spectra. Consequently, the conversion of P-450 to P-420 did not account for the negative results. Assays performed on four different preparations of nuclear membrane from phenobarbital-treated animals indicated the absence of cytochrome P-450. Franke et al. (7) have reported that rat liver nuclear membrane from untreated animals had a cytochrome P-450 content of 0.025 mmole per mg which was one-seventh the level present in the microsomes. In the current study, the microsomal membrane yielded values of 0.62 and 1.57 mmoles of cytochrome P-450 for control and treated animals, respectively (Table I). These values are in agreement with those reported by Estabrook and Cohen (30) but are several orders of magnitude larger than values reported by other investigators (7, 14, 31). It is difficult to make accurate comparisons of specific activity measurements on microsomal constituents conducted in different laboratories since the protein content of the microsomal vesicle can widely vary depending on the method of preparation. It should be emphasized that the microsomes used in this study are disrupted by sonic oscillation and purified by discontinuous gradient centrifugation. This procedure gives predominantly the insoluble membrane matrix free of soluble intravesicular proteins.

Low levels of N-demethylase and aryl hydroxylase activity were found in the nuclear membrane from control animals which corresponded to 11 and 7% of the specific activity obtained for the microsomal membrane, respectively (Table I). A most striking feature was the lack of inducibility of these two enzymes in the nuclear membrane by phenobarbital. This is in marked contrast to the microsomal membrane which exhibited a 2.2-fold increase in N-demethylase activity and a 3.3-fold increase in
The nuclear membrane also contained an enzyme capable of the reductive cleavage of the azo linkage in 3-methyl-4-aminoazobenzene. Quantitative measurements were not made, but the products were detected after thin layer chromatography of the reaction mixture.

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