Characteristics of Benzo(a)pyrene Metabolism and Cytochrome P-450 Heterogeneity in Rat Liver Nuclear Envelope and Comparison to Microsomal Membrane*

(Received for publication, September 12, 1977, and in revised form, November 28, 1977)

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The formation of benzo(a)pyrene metabolites by highly purified nuclear envelope from hepatocytes of control rats was extremely low, and unlike the microsomal system, was not induced by phenobarbital pretreatment. However, after 3-methylcholanthrene pretreatment, metabolism increased in nuclear envelope (60 times) than in microsomes (5.5 times) so that the specific activities (per n mole of cytochrome P-450) equaled. Product distribution was similar between nuclear and microsomal membranes, although a shift toward more phenols was seen concomitant with a decrease in dihydrodiols. This shift relates to the lower levels of epoxide hydratase in the nuclear envelope. The metabolism of benzo(a)pyrene (+)-trans-7,8-dihydrodiol by nuclear envelope was induced by 3-methylcholanthrene treatment (15-fold) and favored formation of the anti diol-oxide stereoisomer (7,8)-7α,8α-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene.

Different control over phenobarbital induction in nuclear envelope and microsomes was shown by other enzymes. Epoxide hydratase activity in nuclear envelope was decreased in animals pretreated with inducers in comparison to the 3-fold induction of the enzyme by phenobarbital in microsomes. NADPH-cytochrome c reductase was also only induced in the microsomes. Four separate forms of cytochrome P-450 were identifiable in the nuclear envelope by their type I difference spectra. The total nuclear envelope cytochrome P-450 remained the same in control and phenobarbital-treated animals; however, phenobarbital administration changed the relative proportions of the forms of cytochrome P-450. In contrast, 3-methylcholanthrene treatment doubled the net nuclear envelope cytochrome P-450 content, shifting the absorbance maximum to 448 nm.

Absence of a microsomal camphor-binding cytochrome P-450 remained the same in control and phenobarbital-treated animals; however, phenobarbital administration changed the relative proportions of the forms of cytochrome P-450. In contrast, 3-methylcholanthrene treatment doubled the net nuclear envelope cytochrome P-450 content, shifting the absorbance maximum to 448 nm. Absence of a microsomal camphor-binding cytochrome P-450 species in nuclear envelope preparations allowed an estimate of less than 1% maximum microsomal contamination.

* This investigation was supported by Grants CA-16265, CA-07175, CA-17300, and Career Development Award CA-00250 (C. R. J.) from the National Cancer Institute, Department of Health, Education and Welfare. The costs of publication of this article were therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of postdoctoral fellowship from the National Institutes of Health.
more significant factor in DNA modification than would be judged simply by its contribution to the total cellular metabolism of benzo(a)pyrene. Metabolism of benzo(a)pyrene by whole nuclei has been measured in several laboratories (18-22); this metabolism has also been correlated with the modification of nuclear DNA. It has been reported that after induction of rats by 3-methylcholanthrene, liver nuclei, and microsomes contributed equally to the modification of nuclear DNA by benzo(a)pyrene metabolites even though metabolism was over 25 times greater by the microsomes (19). However, convincing criteria were not provided in these studies to show that nuclei were free of microsomal contamination. Even a 5 to 10% contamination of nuclei by endoplasmic reticulum can have a dramatic effect on the higher activity of drug metabolism enzymes in this membrane.

In this publication, we describe the metabolism of benzo(a)pyrene by highly purified nuclear envelope preparations which are proven to have less than 1% of microsomal contamination by quantitative, biochemical criteria. For example, in these preparations there is no significant phenobarbital induction of total cytochrome P-450 (15). In other studies of benzo(a)pyrene metabolism, severalfold induction of cytochrome P-450 by phenobarbital is seen in the nuclear preparations. This suggests a significant microsomal contamination in those preparations.

The availability of fully resolved membrane preparations has allowed us to establish that metabolism of benzo(a)pyrene and benzo(a)pyrene 7,8-dihydrodiol are only significant in the nuclear envelope after induction by 3-methylcholanthrene. Different forms of cytochrome P-450 in the nuclear envelope and endoplasmic reticulum are also distinguished.

**EXPERIMENTAL PROCEDURES**

Male Holstein rats weighing 50 to 60 g were used in all experiments. Inductions by both phenobarbital and 3-methylcholanthrene were done by previously described procedures (17). Hepatic nuclear envelope was isolated and prepared as previously described (23, 24). Liver microsomes were prepared by the method of van der Hoeven et al. (25). Protein concentrations were determined by the Folin procedure (26) using crystallized ovalbumin as the protein standard.

**Hydrocarbon Metabolism**—The formation of benzo(a)pyrene metabolites was examined using incubation conditions previously described (27). [3H]Benzo(a)pyrene (8.3 Ci/mmol) was purified by high pressure liquid chromatography shortly before use. The specific activity of the benzo(a)pyrene substrate used in incubations containing microsomes or 3-methylcholanthrene nuclear envelope was 20.0 Ci/mmol, and for control and phenobarbital nuclear envelope incubations was 400 Ci/mmol. Reactions were for 30 min at 37°C; extraction on a Vortex mixer. After adding the substrate, the foil-wrapped tubes were shaken for 15 min at 37°C; the reaction was stopped by adding 2.5 ml of acetone-acetyl acetate (1:2, 1% triethylamine), followed by extraction on a Vortex mixer. After adding MgSO4 and centrifuging, 2.0 ml of the organic phase was transferred and evaporated. The residue was dissolved in methanol and then analyzed by high-pressure liquid chromatography on an ODS Zorbax column (methanol:water (90:10) at 1.0 ml/min). BP 4,5-dihydrodiol was quantitated relative to a standard amount of benzo(a)pyrene by electronic peak integration.

**Epoxide Hydrolase Activity**—The epoxide hydrolytic activity of the membranes was measured by the method of Nebert and Gelboin (28). The anionic reduction of BP 4,5-oxide by 3-methylcholanthrene nuclear envelope was measured by previously described techniques (27).

**Total cytochrome P-450 Content**—Cytochrome P-450 content was measured by the method of Omura and Sato (29). Liver microsomes were measured with a DW2 spectrophotometer (American Instrument Co.) which was operated either in the split beam or dual wavelength mode. SKF 525A, lidocaine (both in water), and testosterone (2 mg/ml in acetone) were added sequentially to membrane suspensions, and the substrate was added. The substrate was added, and the tubes shaken for 6 min. Eight milliliters of acetone-acetyl acetate (1:2, 1% triethylamine) was quickly added; after extraction on a Vortex mixer and centrifuging, 6 ml of solvent was transferred and evaporated. The residue was dissolved in methanol and then analyzed by high-pressure liquid chromatography on an ODS Zorbax column (methanol:water (90:10) at 1.0 ml/min). BP 4,5-dihydrodiol was quantitated relative to a standard amount of benzo(a)pyrene by electronic peak integration.

1 Liver microsomes or nuclear envelope from rats which have been induced by 3-methylcholanthrene microsomes. [3H]BP 7,8-dihydrodiol was likewise produced in 2-ml incubations containing [3H]benzo(a)pyrene. Pure product was isolated by preparative high pressure liquid chromatography using an ODS Zorbax column (6.2 mm x 0.25 m). The BP 7,8-dihydrodiol specific activity was 100 Ci/mmol in incubations containing microsomes or 3-methylcholanthrene nuclear envelope and was 1.24 Ci/mmol for control nuclear envelope incubations. BP 7,8-dihydrodiol metabolites were formed in a 0.5-ml incubation containing: 0.25 to 2.0 mg of protein, 0.03 unit of glucose-6-phosphate dehydrogenase, 3.5 μmol of glucose-6-phosphate, 0.95 μmol of NADP, 1.5 μmol of MgCl2, 25 μmol of potassium phosphate buffer (pH 7.5), and 5 or 20 nmol of [3H]BP 7,8-dihydrodiol added in 25 μl of acetone. After adding the substrate, the foil-wrapped tubes were shaken for 15 min at 37°C; the reaction was stopped by adding 2.5 ml of acetone-acetyl acetate (1:2, 1% triethylamine), followed by extraction on a Vortex mixer. After adding MgSO4 and centrifuging, 2.0 ml of the organic phase was transferred and evaporated at 45°C under a stream of nitrogen.

2 The abbreviations used are: BP 7,8-dihydrodiol, trans-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; BP 7,8-dihydrodiol 9,10-oxide, (+)-7a,8p-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; BP 4,5- and 9,10-dihydrodiol, benzo(a)pyrene 4,5-oxide and 9,10-oxide, either or both stereoisomers; tetrol, tetrahydroxytetrahydroepoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; SKF 525A, N,N-diethylaminomethyl diphenylpropyl acetate.
Five-milliliter reaction mixtures containing the indicated amount of protein were incubated for 30 min at 37°C as described under "Experimental Procedures." Benzo(a)pyrene concentration was either 10 μM (control and PB-nuclear envelope), 20 μM (MC-nuclear envelope and MC-microsomes), 17 μg/ml, or 60 μM (microsomes, 150 μg/ml). Activities were corrected using blank tubes containing heat-inactivated microsomal membrane.

<table>
<thead>
<tr>
<th>Enzyme content</th>
<th>control, phenobarbital- and 3-methylcholanthrene-induced rat liver nuclear envelopes, and microsomal membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme source</td>
<td>Dihydrodiols</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Control nuclear envelope</td>
<td>9.1</td>
</tr>
<tr>
<td>300 μg/ml</td>
<td>(13 ± 3)</td>
</tr>
<tr>
<td>Control microsomes</td>
<td>1,844</td>
</tr>
<tr>
<td>400 μg/ml</td>
<td>(10.6</td>
</tr>
<tr>
<td>PB nuclear envelope</td>
<td>32 ± 10</td>
</tr>
<tr>
<td>300 μg/ml</td>
<td>(10 ± 5</td>
</tr>
<tr>
<td>PB microsomes</td>
<td>1,860</td>
</tr>
<tr>
<td>400 μg/ml</td>
<td>(4.5</td>
</tr>
<tr>
<td>MC nuclear envelope</td>
<td>2,017 ± 447</td>
</tr>
<tr>
<td>80 μg/ml</td>
<td>(11 ± 3</td>
</tr>
<tr>
<td>MC microsomes</td>
<td>160</td>
</tr>
<tr>
<td>150 μg/ml</td>
<td>(27.9)</td>
</tr>
<tr>
<td>17 μg/ml</td>
<td>11,295</td>
</tr>
<tr>
<td>(11.8)</td>
<td>(11.0)</td>
</tr>
</tbody>
</table>

- Mean and standard deviation of four preparations (CO, MC) and six preparations (PB).
- Single metabolite as a percent of the total metabolites formed in an incubation, mean and standard deviation.
- Mean of duplicate determinations.
- Not determined.

decrease in the proportion of BP 9,10-dihydrodiol and an increase in BP 9-phenol as compared to microsomal metabolism. A similar trend has been reported by Jernstrom et al. in comparing intact nuclei and microsomes (20). This change in product distribution is seen, however, when the level of protein is decreased from 150 to 17 μg/ml in the 3-methylcholanthrene microsomal membranes which may indicate changes in the characteristics of the cytochrome P-450 between nuclear envelope and microsomes. On the other hand, 3-methylcholanthrene caused a higher induction of benzo(a)pyrene metabolism and epoxide hydratase levels are comparable to those found in nuclear envelope incubations.

The total activities, cytochrome P-450 contents, and activities per unit cytochrome P-450 are compared in Table II. Phenobarbital failed to induce either total metabolism of benzo(a)pyrene or the content of cytochrome P-450 in the nuclear envelope. On the other hand, 3-methylcholanthrene caused a higher induction of benzo(a)pyrene metabolism and a similar induction of cytochrome P-450 in the nuclear envelope as compared to the corresponding changes in microsomes. The specific activity for benzo(a)pyrene metabolism per unit of cytochrome P-450 was the same in both microsomes and nuclear envelope after 3-methylcholanthrene induction. The λox values for the reduced carbon monoxide difference spectra were similarly shifted to near 448 nm in both nuclear envelope and microsomes after 3-methylcholanthrene induction, while this peak remained at 450 nm or longer wavelengths for control or phenobarbital-induced preparations. There was a distinction in λox values between microsomal and nuclear envelope preparations for control and phenobarbital treatments which may indicate changes in the characteristics of the cytochrome P-450 between nuclear envelope and microsomes.

Epoxide hydratase activities were measured in each membrane preparation using BP 4,5-oxide as substrate. Nuclear envelope activities were not susceptible to induction, most notably by phenobarbital which induced microsomal activity 3-fold (Table III). Indeed, a significant decrease in nuclear activity was observed after pretreatments by 3-methylcholanthrene and phenobarbital. Nuclear epoxide hydratase activity was only one-half of microsomal activity in control animals while there was an almost 3-fold decrease in specific cytochrome P-450 content. Jernstrom et al. (20) report decreases in whole nuclei relative to microsomes of 48 times for epoxide hydratase and 37 times for cytochrome P-450. The most mutagenic metabolites of benzo(a)pyrene are the syn and anti BP 7,8-dihydriodiol 9,10-oxides derived from the further oxidation of BP 7,8-dihydriodiol. It has been shown that microsomal formation of BP 7,8-dihydriodiol is stereospecific for one optical isomer of BP 7,8-dihydriodiol, while chemical synthesis yields a racemic mixture (7). The syn and anti
forms have been quantitated by high pressure liquid chromatography via their tetrol hydrolysis and triol reduction products (7, 30) (Fig. 1) using enzymatically formed BP 7,8-dihydrodiol as the substrate (Table IV). The ratio of tetrols formed by hydrolysis of pure enantiomers and the relative retention times were similar to those reported by Yang et al. (7). Two incubation conditions were found to be necessary because of the exceedingly low activity of control nuclear envelope. Metabolism of BP 7,8-dihydrodiol was determined in control nuclear envelope with a low substrate concentration (10 μM) and a high protein concentration (4.6 mg/ml). The enzyme is far from saturated with substrate, but percentage conversion is maximized. 3-Methylcholanthrene-induced nuclear envelope was incubated under these conditions with a shorter reaction time so as to prevent excessive substrate conversion. 3-Methylcholanthrene-induced nuclear envelope was also incubated under substrate saturation conditions (40 μM) and a high protein concentration (4.6 mg/ml). The enzyme is far from saturated with substrate, but percentage conversion is maximized.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>BP 4,5-oxide hydration nmol/mg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.32 ± 0.41*</td>
</tr>
<tr>
<td>MC</td>
<td>3.07 ± 0.21*</td>
</tr>
<tr>
<td>PB</td>
<td>3.64 ± 0.30*</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.34 ± 0.52</td>
</tr>
<tr>
<td>MC</td>
<td>11.61 ± 0.94</td>
</tr>
<tr>
<td>PB</td>
<td>29.93 ± 3.34</td>
</tr>
</tbody>
</table>

* Mean and standard deviation of at least four determinations.

** Significantly different from control, p < 0.01.

The type I spectral response produced by saturation of the binding with a ligand is approximately proportional to the microsomal content of that form of cytochrome P-450 providing endogenous substrates do not occupy the sites. The selectivity of these ligands for particular forms of cytochrome P-450 means that the ligands can be added sequentially, in the other shown in Table V. In amounts sufficient to nearly saturate the appropriate site without affecting the spectral responses at other sites. For simplicity, the two microsomal lidocaine sites have not been distinguished in Table V. Powis et al. have recently shown that endogenous substrates are substantially depleted by the addition of defatted bovine serum albumin to the medium (36) and this approach has been used in this study. Cytochrome P-450 (P-448) which is induced by polycyclic hydrocarbons apparently adopts a predominantly high spin state even in the absence of substrate and does not...

\[ \text{Fig. 1. High pressure liquid chromatographic analysis of hydrolysis products derived from synthetic dihydrodiol-oxides: anti stereoisomer (---), syn stereoisomer (---). Pure dihydrodiol-oxide stereoisomers were added to separate, standard incubations for 15 min ("Experimental Procedures"). The extracted hydrolysis products were then resolved by high pressure liquid chromatography. For metabolism experiments, products of \(^{13} \text{H} \)BP 7,8-dihydrodiol and unreacted dihydrodiol were separated and collected in 0.3-min fractions (0 to 20 min) or 1.0-min fractions (20 to 40 min).} \]
Cytochrome P-450 in the nuclear envelope. Cytochrome P-450 effects of inducers. One per cent bovine serum albumin inhibited and lidocaine but had no detectable effect on the binding of SKF 525A (small response). Lidocaine, and testosterone. The increased microsomal responses to high concentrations of camphor and lidocaine but had no detectable effect on the binding of type I ligands. However, in control nuclear envelope (Fig. 2A), the major response was to testosterone, while after phenobarbital induction the major response was to lidocaine (Fig. 2B). Thus, phenobarbital induction changes the relative proportion of cytochrome P-450 indicating that all cytochromes P-450 give a type I response produced by each ligand addition.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>NE</th>
<th>MIC</th>
<th>NE</th>
<th>MIC</th>
<th>NE</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKF 525A</td>
<td>5 ± 1 (10)</td>
<td>115 (33)</td>
<td>7 ± 1 (27)</td>
<td>120</td>
<td>9 ± 3 (18)</td>
<td>350 (29)</td>
</tr>
<tr>
<td>Camphor (20 μM)</td>
<td>&lt;3</td>
<td>8 (2)</td>
<td>&lt;3</td>
<td>10</td>
<td>&lt;3</td>
<td>125 (10)</td>
</tr>
<tr>
<td>Lido- caine (200 μM)</td>
<td>5 ± 2 (10)</td>
<td>60 (17)</td>
<td>&lt;3</td>
<td>95</td>
<td>26 ± 6 (53)^d</td>
<td>410 (34)</td>
</tr>
<tr>
<td>Camphor (650 μM)</td>
<td>&lt;3</td>
<td>80 (25)</td>
<td>&lt;3</td>
<td>30</td>
<td>&lt;3</td>
<td>245 (20)</td>
</tr>
<tr>
<td>Testosterone (90 μM)</td>
<td>39 ± 3 (80)</td>
<td>80 (23)</td>
<td>19 ± 4 (73)^d</td>
<td>90</td>
<td>14 ± 5 (28)^d</td>
<td>70 (6)</td>
</tr>
<tr>
<td>Total Type I</td>
<td>49</td>
<td>343</td>
<td>26</td>
<td>345</td>
<td>49</td>
<td>1200</td>
</tr>
<tr>
<td>Reduced CO</td>
<td>165 ± 45</td>
<td>556</td>
<td>340 ± 50</td>
<td>1580</td>
<td>220 ± 44</td>
<td>2184</td>
</tr>
</tbody>
</table>

As determined by similarity in retention, the above peaks correspond to the following hydrolysis products as determined by Yang et al. (7): I, (7,10/8,9)-tetrol; III, (7/8,9,10)-tetrol; V, (7/8,9)-tetrol.

### Table IV

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>U^- I derivatives</th>
<th>syn derivatives</th>
<th>Total</th>
<th>Ratio^e anti</th>
<th>Total BP 7,8-dihydrodiol oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear envelope</td>
<td>2.9</td>
<td>±0.1</td>
<td>20.2</td>
<td>±4.1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>10 μM substrate</td>
<td>120</td>
<td>±100</td>
<td>290</td>
<td>±140</td>
<td>34</td>
</tr>
<tr>
<td>Microsomes</td>
<td>100</td>
<td>±100</td>
<td>1,400</td>
<td>±140</td>
<td>35</td>
</tr>
<tr>
<td>Control</td>
<td>42</td>
<td>±100</td>
<td>420</td>
<td>±140</td>
<td>58</td>
</tr>
<tr>
<td>40 μM substrate</td>
<td>920</td>
<td>±100</td>
<td>8,800</td>
<td>±140</td>
<td>940</td>
</tr>
</tbody>
</table>

^e Unknown derivative eluting immediately before Fraction I.
^f The sum of Fractions I, III, and V over II and IV.
^g Mean and standard deviation of triplicate determinations.
^h Either 5 or 20 nmol of [3H]BP 7,8-dihydrodiol were added to 0.5 ml incubations in 25 μl of acetone.

### Table V

Type I ligand-binding to P-450 cytochromes in nuclear envelope and microsomal membrane

Ligands were added in sequence (Compounds 1 through 5) to 1-ml incubations containing 1.2 mg/ml of protein. Numbers show the increase in spectral response from each addition (AA:,,,,,,,, ). Nuclear envelope values are the mean of four determinations, microsomal enzyme source: MIC, microsomes. Numbers in parentheses indicate percentage of total type I response produced by each ligand addition.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Δ (absorbance) x 10^4 mg^-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>MIC</td>
<td>NE</td>
</tr>
<tr>
<td>1. SKF 525A (3 μM)</td>
<td>5 ± 1 (10)</td>
<td>115 (33)</td>
</tr>
<tr>
<td>2. Camphor (20 μM)</td>
<td>&lt;3</td>
<td>8 (2)</td>
</tr>
<tr>
<td>3. Lidocaine (200 μM)</td>
<td>5 ± 2 (10)</td>
<td>60 (17)</td>
</tr>
<tr>
<td>4. Camphor (650 μM)</td>
<td>&lt;3</td>
<td>80 (25)</td>
</tr>
<tr>
<td>5. Testosterone (90 μM)</td>
<td>39 ± 3 (80)</td>
<td>80 (23)</td>
</tr>
<tr>
<td>Total Type I</td>
<td>49</td>
<td>343</td>
</tr>
<tr>
<td>Reduced CO</td>
<td>165 ± 45</td>
<td>556</td>
</tr>
</tbody>
</table>

^a ΔA_0-30s.
^b ΔA_180-360s.
^c In microsomes, this response corresponds to high and low affinity sites.
^d Spectral response significantly different from control nuclear envelope. p < 0.01.

Exhibit type I spectral interactions. Thus, after induction by 3-methylcholanthrene, a substantial proportion of cytochrome P-450 does not respond to type I ligands. However, in control and phenobarbital microsomes, the ratio of type I to reduced CO responses is typical of purified microsomal cytochrome P-450 indicating that all cytochromes P-450 give a type I response to one of the ligands.

Cytochrome P-450 in the liver nuclear envelope clearly showed different specificity for type I ligands than cytochrome P-450 of liver microsomes and responded differently to the effects of inducers. One per cent bovine serum albumin increased microsomal responses to high concentrations of camphor and lidocaine but had no detectable effect on the binding to cytochrome P-450 in the nuclear envelope. Cytochrome P-450 in the nuclear envelope exhibited type I binding to only SKF 525A (small response), lidocaine, and testosterone. The response to each ligand was not affected by the presence of the other two ligands and therefore, each response corresponds to an interaction with a distinct form of cytochrome P-450.

Major differences in these responses in the nuclear envelope were observed after induction by phenobarbital or 3-methylcholanthrene (Table V). In control nuclear envelope (Fig. 2A), the major response was to testosterone, while after phenobarbital induction the major response was to lidocaine (Fig. 2B). Thus, phenobarbital induction changes the relative proportions of individual cytochrome P-450 species without increasing the total cytochrome P-450 content. 3-Methylcholanthrene induction caused only a decrease in the testosterone response while doubling the total cytochrome P-450. This is consistent with an increase in cytochrome P-448, which, as a high spin cytochrome, does not undergo type I interactions.

Lidocaine binds to high affinity (K_i = 3.6 μM) and low
affinity \(K_o = 35 \mu M\) sites in phenobarbital-induced liver microsomes but to only one site \(K_o = 6.1 \mu M\) in phenobarbital-induced nuclear envelope. However, the insensitivity of lidocaine binding in the nuclear envelope to SKF 525A (50 \mu M) and testosterone (90 \mu M) distinguished this form of cytochrome P-450 from the form in phenobarbital-induced microsomes with the higher affinity for lidocaine.

A comparison of type I interactions with cytochrome P-450 in nuclear envelope and microsomes indicates several differences. There were relatively much lower responses to SKF 525A and camphor in the nuclear envelope. By comparison of these spectral responses, we can place a limit on microsomal contamination of the nuclear envelope (1% phenobarbital-induced, 3% 3-methylcholanthrene-induced, 4% control). In microsomes, testosterone competes for the binding sites on cytochrome P-450 which are occupied by SKF 525A, camphor, and lidocaine. In the nuclear envelope, testosterone binding is restricted to the steroid-specific form. The ratio of the total type I response to the reduced carbon monoxide response or cytochrome P-450 of the nuclear envelope is less than half that found for the microsomes. This suggests that ligands which bind to the substrate site of some nuclear envelope cytochrome P-450 species remain unknown.

**DISCUSSION**

The nuclear envelope, which is contiguous with the endoplasmic reticulum, contains many of the same biological activities, notably cytochrome P-450 (12-14), NADPH-cytochrome c reductase (12, 13, 15, 16), epoxide hydratase, and UDP-glucuronosyltransferase (37). Detailed comparative studies of the distribution of these enzymes require the use of highly purified nuclear envelope. Since the mass of nuclear envelope in the cell is approximately 1% of the total mass of microsomal membrane protein, the opportunity for serious contamination is quite significant. Envelope prepared by the discontinuous sucrose-citrate density gradient procedure (23, 24) has been characterized extensively (13, 14, 17, 23, 24, 38, 39) and shown to possess many of the chemical and biochemical features of the endoplasmic reticulum. Definite qualitative differences between these two closely associated membrane systems do exist. For example, specific nuclear envelope enzymes are refractive to induction by phenobarbital. Included in this group are cytochrome P-450, NADPH-cytochrome c reductase, aryl hydroxylase, N-demethylase, and epoxide hydratase. Also UDP-glucuronosyltransferase, which glucuronidates hydroxylated products arising from the NADPH electron transport chain, is not induced. In the case of each of the above proteins, the corresponding activity in the endoplasmic reticulum is induced by phenobarbital. A second rigorous criterion establishing the absence of contamination by endoplasmic reticulum is based upon the occurrence of an endogenous protein kinase in the endoplasmic reticulum that phosphorulates intrinsic microsomal membrane proteins not found in the nuclear envelope. Incubation of highly purified nuclear envelope under conditions optimal for phosphorylation yields no \(^{32}\)P-labeled proteins characteristic of the microsomal membrane when subjected to autoradiographic analysis. Thus, these two methods provide extremely sensitive means for evaluation of possible contamination of nuclear envelope preparations by endoplasmic reticulum. A quantitative comparison of cytochrome P-450 and phosphorylation patterns in the nuclear envelope and microsomes allows us to then place a limit of approximately 1% on the contamination of nuclear envelope with microsomes by two independent techniques.

The specific activity per unit of cytochrome P-450 and the product distribution for metabolism of benzo(a)pyrene provide valuable criteria for determining the nature of P-450 cytochromes in these membranes. The low level of benzo(a)pyrene metabolism activity in control and the further reduction of this activity in phenobarbital-induced nuclear membrane (per nmol of cytochrome P-450) relative to microsomal activities, indicates differences in the complement of P-450 cytochromes in nuclear membranes as compared to endoplasmic reticulum. Although the nuclear envelope contains approximately one-third less NADPH-cytochrome c reductase than the microsomal membrane (14), benzo(a)pyrene oxidation expressed per nanomole of cytochrome P-450 is the same for 3-methylcholanthrene-induced nuclear envelope and microsomal membrane. It seems, therefore, that a decrease in NADPH-cytochrome c reductase activity in the nuclear envelope is not responsible for the low activities of control and phenobarbital-induced nuclear envelope.

The general similarity of product distributions for benzo(a)pyrene metabolism in the nuclear envelope and microsomes implies that the forms of cytochrome P-450 which catalyze the oxygenation of benzo(a)pyrene are nearly the

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4 J. Gorski and C. B. Kasper, manuscript in preparation.
5 K. Lam and C. B. Kasper, manuscript in preparation.
same in both membranes, irrespective of inducer but that of microsomal incubations. Product distributions were closely matched (Table I). Stable BP 7,8-oxide is most affected by this change. When cytochrome P-450 and epoxide hydratase levels were comparable in 3-methylcholanthrene-induced nuclear envelope and microsomes, product distributions were closely matched (Table I).

In all discussions of the multiplicity of cytochrome P-450 the word "form" is used loosely. That a given set of properties correspond to a single homogenous cytochrome P-450 can only be rigorously proven by purification to the point where a single peptide chain is demonstrated. The term "form of cytochrome P-450" is used generally to mean a category of one or more P-450 cytochromes distinguished from other categories by one or more properties. Frequently molecular weight (gel electrophoresis) (33) or immunoreactivity (40) are used while here ligand-binding specificity has proven to be the most accessible property.

Analysis of cytochrome P-450 in the nuclear envelope and microsomes by means of type I difference spectra (Table V) indicates substantial differences between the forms of cytochrome P-450 in the two membranes. Four forms of P-450 cytochrome can be clearly discerned in the nuclear membrane with appropriate pretreatments of the rats. One form which binds lidocaine is only observable after phenobarbital induction, and can be distinguished from lidocaine-responding forms in the microsomes by a distinct $K_r$ and Type I site which is insensitive to SKF 525A and testosterone. A second form is detected by a type I response to testosterone and is insensitive to the presence of ligands such as SKF 525A, lidocaine, and camphor. This form decreases when the rats are exposed to induction by either phenobarbital or 3-methylcholanthrene. A form with similar binding characteristics was observed in microsomes which was also insensitive to induction. Although the absolute amount of nuclear envelope cytochrome P-450 is not increased by phenobarbital induction, there is clearly an induction of one form which binds lidocaine and a decrease of another form which binds testosterone. Hence, phenobarbital administration markedly affects the levels of individual species of nuclear P-450 without producing a net increase.

3-Methylcholanthrene induces cytochrome P-448 in the nuclear envelope as has been previously noted (20, 22), although the possible influence of microsomal contamination in these preparations was not determined. This cytochrome seems to be similar to the microsomal cytochrome as evidenced by the same activity and product distribution in the metabolism of benzo(a)pyrene. A small proportion of nuclear envelope cytochrome P-450 binds SKF 525A with high affinity ($K_r < 1 \mu M$) but it should be noted that a similar form is a major contributor to microsomal cytochrome P-450, particularly from control animals. Bresnick et al. have reported that SKF 525A has less effect on drug metabolism in the nuclear envelope than in the microsomes (22). Forms which can be detected in microsomes by means of type I responses to camphor were completely absent in the nuclear envelope and have been used above as the basis for setting a limit to microsomal contamination in the nuclear envelope. The nuclear envelope, unlike the microsomes, contained negligible amounts of endogenous substrates which could be removed by bovine serum albumin (36). On the other hand, the metyrapone difference spectrum in Fig. 2B indicates some high spin cytochrome P-450 in the control nuclear envelope (41).

The small proportion of nuclear envelope cytochrome P-450 which can be detected by the type I ligands, just as with 3-methylcholanthrene microsomes, is a result of the high content of P-448, since this form does not show type I spectral responses. It also seems likely on the basis of the ratio of type I to reduced CO spectral responses (Table V) that only about half of the nuclear envelope cytochrome P-450 is detectable by the selected type I ligands while essentially all of the microsomal cytochrome P-450 is responsive.

The amount of the highly mutagenic BP 7,8-dihydrodiol 9,10-oxide formed within the nuclear envelope after 3-methylcholanthrene induction suggests that the nuclear membrane may be important in determining conjugation to DNA by these metabolites. Total binding of benzo(a)pyrene to DNA within liver nuclei per unit of cytochrome P-450 or per unit of benzo(a)pyrene metabolism has been found to be somewhat higher after 3-methylcholanthrene induction from nuclear enzymes than from added liver microsomes (19). The short half-life of the BP 7,8-dihydrodiol 9,10-oxides (42, 43) and the proximity to DNA may account for this enhanced effectiveness of the nuclear membrane.

To evaluate the stereospecificity of oxygenation of BP 7,8-dihydrodiol in the nuclear envelope, the (−)-trans-7,8-dihydrodiol was prepared enzymatically by the stereospecific oxygenation of benzo(a)pyrene, as has previously been described (7). Oxidation at the 9,10 position of BP 7,8-dihydrodiol leading to formation of the anti stereoisomer was favored by both control and 3-methylcholanthrene-induced nuclear envelope by a factor of two. With more nearly saturating amounts of substrate, this preference increased inexplicably to 3.2 times. This same trend has been reported for the microsomal reaction (30). The anti/syn ratio for reaction with 3-methylcholanthrene microsomes is in reasonable agreement with the value (2.6) reported by Yang et al. (7). Metabolism of BP 7,8-dihydrodiol, like benzo(a)pyrene, was very low in control nuclear envelope but induction by 3-methylcholanthrene was about 20-fold. Thus, induction of the metabolism of BP 7,8-dihydrodiol broadly follows that of benzo(a)pyrene in both nuclear envelope and microsomes although there was some indication of differences between the two membranes for metabolism of these compounds. This work establishes that metabolism of benzo(a)pyrene and BP 7,8-dihydrodiol in the nuclear envelope is only likely to be physiologically relevant after induction by 3-methylcholanthrene and presumably other inducers which affect the Ah locus (44).

Like cytochrome P-450 and NADPH-cytochrome P-450 reductase, epoxide hydratase activity in the purified nuclear membrane was not induced by phenobarbital. Indeed, small but significant decreases in epoxide hydratase activity were observed after pretreatment of the rats with 3-methylcholanthrene or phenobarbital. This may be related to decreases in the steroid-binding form of cytochrome P-450 in the nuclear envelope from these treatments.

The availability of purified nuclear membranes has permitted clear demonstration that induction of nuclear membrane
enzymes is specifically associated with expression of genes at the Ah issue. The failure of phenobarbital to increase the levels of enzymes in the nuclear membrane which are increased in the endoplasmic reticulum remains an important clue to intracellular mechanisms for distribution of newly synthesized enzymes. The high level of induction of cytochrome P-448 and polycyclic hydrocarbon metabolism in the nuclear membrane seen as a result of polycyclic hydrocarbon exposure may contribute significantly to cell transformation during long term exposure to these compounds.

Acknowledgment — We wish to thank John Sheehan for his expert technical assistance.

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