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 more in nuclear envelope (60 times) than in microsomes (5.5 times) so that the specific activities (per nmol of cytochrome P-450) equalized. 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-dihydriodiol by nuclear envelope was induced by 3-methylcholanthrene treatment (15-fold) and favored formation of the anti diol-oxide stereoisomer. (\(+\)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene).

Differential 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 species in nuclear envelope preparations allowed an estimate of less than 1% maximum microsomal contamination.

The covalent binding of polycyclic aromatic hydrocarbon and of many other molecules to DNA requires an initial metabolism of the compound via a mixed function oxidation system which is dependent on cytochrome P-450. This process has been implicated as an essential initial step in the carcinogenic activity of polycyclic aromatic hydrocarbons. The metabolism of one such hydrocarbon, benzo(a)pyrene, via the cytochrome P-450 system of the liver endoplasmic reticulum has been studied extensively (2-4). Separation of benzo(a)pyrene metabolites by high pressure liquid chromatography has indicated the formation of dihydrodiols, quinones, phenols, oxides, and dihydrodiol-oxides (5-7). Benzo(a)pyrene 4,5,7,8, and 9,10-oxides are converted to the corresponding trans-dihydriodiol by microsomal epoxide hydratase while further oxygenation of benzo(a)pyrene 7,8-dihydriodiol to stereoisomeric benzo(a)pyrene 7,8-dihydriodiol 9,10-oxides has also been shown (7). All of these benzo(a)pyrene oxides and dihydrodiol-oxides have been shown to be active mutagens (8, 9), although the benzo(a)pyrene 7,8-dihydriodiol 9,10-oxides have proved to be by far the most potent. Covalent binding of these oxides to DNA bases has been established (10, 11). However, the great reactivity of most of these oxides also leads to rapid reaction with water which then competes with DNA for these intermediates.

Cytochrome P-450 (12-14) and NADPH-cytochrome c reductase (12, 13, 15, 16) have been found in purified nuclear envelope, and this membrane has been shown to support metabolism of benzo(a)pyrene (13, 17). The nuclear membrane has a bi-leaflet structure in which the outer membrane forms a continuum with the endoplasmic reticular membranes. Nuclear envelope preparations can be distinguished from endoplasmic reticulum by the lower activities of cytochrome P-450, NADPH-cytochrome c reductase, and drug metabolism in the nuclear envelope and by the complete failure of phenobarbital to induce these activities in this membrane (13, 14). In contrast, the metabolism of benzo(a)pyrene is induced in the nuclear envelope by 3-methylcholanthrene to a much greater extent than in the endoplasmic reticulum (17).

The relative instability of mutagenic benzo(a)pyrene oxides and dihydrodiol-oxides implies that proximity of the site of generation of these metabolites to the site of DNA modification may be significant in determining the extent and nature of the DNA alteration. This proximity factor may make metabolism of benzo(a)pyrene in the nuclear membrane a
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 rate of 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 (13). 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-dihydridiol 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 recrystallized ovalbumin as the protein standard.

Hydrocarbon Metabolism - The formation of benzo(a)pyrene metabolites was examined using incubations 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 envelopes 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 and high pressure liquid chromatography analysis of the metabolites were done as previously described (27). Unlabeled metabolite standards were added to the radioactive products prior to analysis.

Optically active BP 7,8-dihydridiol was synthesized in 150 ml incubations containing 100 μM benzo(a)pyrene and 300 mg of 3-methylcholanthrene microsomes. [3H]BP 7,8-dihydridiol 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-dihydridiol specific activity was 100 Ci/mmol in incubations containing microsomes or 3-methylcholanthrene nuclear envelopes and was 1.74 Ci/mmol for control nuclear envelope incubations. BP 7,8-dihydridiol 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, 0.05 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-dihydridiol added in 0.5 ml 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 acetonitrile (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 nitrogen stream. The products were then analyzed by high pressure liquid chromatography on a DuPont 845 instrument ODS Zorbax column (6.2 mm x 0.25 m). 80-min linear gradient of 60 to 100% methanol water at ambient temperature, flow rate was 1.1 ml/min). Fractions were collected every 0.3 min for the first 20 min of the chromatogram and every 1.0 min for the final 20 min. In separate experiments, pure anti and syn BP 7,8-dihydridiol 9,10-oxides (60.4 μg in 0.2 ml of acetone NH2OH, 100U:1) were added to standard 5 ml incubations containing 2.5 mg of 3-methylcholanthrene microsomes. High pressure liquid chromatography analysis of these extracts yielded retention times for specific tetrol and tetrahydatin derivatives of each stereoisomeric dihydridiol-oxide.

Epoxide hydratase activity was measured in a 2-ml incubation containing: 0.3 mg of protein, 100 μmol of potassium phosphate buffer (pH 9.0), and 300 nmol of BP 4,5-oxide added in 0.1 ml of acetone. After a 5 min pretreatment at 37°C, the substrate was added and the tubes shaken for 6 min. Eight milliliters of acetonitrile (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-dihydridiol was quantitated relative to a standard amount of benzo(a)pyrene by electronic peak integration.

Aryl hydroxylase activity was measured by the method of Nebert and Gelboin (28). The anaerobic reduction of BP 4,5-oxide by 3-methylcholanthrene nuclear envelope was measured by previously described techniques (27).

Total cytochrome P-450 content was measured by the method of Oesch and Soto (29). Liver nuclear membranes were assayed 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. The response to each ligand was not affected by the presence of the other ligands. SKF 525A was a gift of Smith, Kline and French Inc., standard benzo(a)pyrene metabolites were obtained from the NCI Chemical Repository, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis).

RESULTS

In Table 1 the distribution of products for metabolism of benzo(a)pyrene by liver nuclear membranes and microsomes are compared. Although the specific activity per mg of protein or oxygenation of benzo(a)pyrene is much lower in the nuclear envelopes, the distribution of the dihydridiol products is similar to that found in microsomes. This product distribution is a characteristic of the forms of cytochrome P-450 which catalyze the oxygenation reaction and is clearly different between control, 3-methylcholanthrene-induced and phenobarbital-induced preparations. In incubations containing 3-methylcholanthrene nuclear envelope, there is a noticeable...
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 of nuclear and microsomal membranes from control, MC- and PB-induced animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme source</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Nuclear envelope</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>PB</td>
</tr>
<tr>
<td>MC</td>
</tr>
<tr>
<td>Microsomes</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>PB</td>
</tr>
<tr>
<td>MC</td>
</tr>
</tbody>
</table>

* Mean and standard deviation of values from at least four preparations of nuclear envelope or mean from two preparations of microsomes.

5,800 Nuclear Envelope Mixed Function Oxidase System

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 source</th>
<th>Dihydrodiols</th>
<th>Quinones</th>
<th>Phenols</th>
<th>Total activity</th>
<th>Aryl hydrocarbon oxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg</td>
<td>pmol/mg</td>
<td>pmol/mg</td>
<td>pmol/mg</td>
<td>pmol/mg</td>
</tr>
<tr>
<td>Control nuclear envelope</td>
<td>37 ± 19</td>
<td>51 ± 23</td>
<td>6 ± 3</td>
<td>118 ± 67</td>
<td>12 ± 7</td>
</tr>
<tr>
<td>300 μg/ml</td>
<td>(13 ± 3)³</td>
<td>(15 ± 4)</td>
<td>(3 ± 1)</td>
<td>(38 ± 7)</td>
<td>(4 ± 1)</td>
</tr>
<tr>
<td>Control microsomes</td>
<td>1,844</td>
<td>4,734</td>
<td>2,228</td>
<td>2,708</td>
<td>299</td>
</tr>
<tr>
<td>400 μg/ml</td>
<td>(10.6)</td>
<td>(27.1)</td>
<td>(12.8)</td>
<td>(15.5)</td>
<td>(17.1)</td>
</tr>
<tr>
<td>PD nuclear envelope</td>
<td>32 ± 10</td>
<td>134 ± 62</td>
<td>&lt;3</td>
<td>28 ± 46</td>
<td>&lt;3</td>
</tr>
<tr>
<td>300 μg/ml</td>
<td>(10 ± 5)</td>
<td>(42 ± 15)</td>
<td>(30 ± 7)</td>
<td>(9 ± 4)</td>
<td></td>
</tr>
<tr>
<td>PB microsomes</td>
<td>1,860</td>
<td>21,513</td>
<td>4,407</td>
<td>6,990</td>
<td>302</td>
</tr>
<tr>
<td>400 μg/ml</td>
<td>(4.6)</td>
<td>(73.3)</td>
<td>(10.9)</td>
<td>(11.2)</td>
<td>(1.0)</td>
</tr>
<tr>
<td>MC nuclear envelope</td>
<td>2,017 ± 477</td>
<td>1,725 ± 237</td>
<td>2,347 ± 736</td>
<td>3,338 ± 780</td>
<td>2,729 ± 302</td>
</tr>
<tr>
<td>80 μg/ml</td>
<td>(11 ± 3)</td>
<td>(10 ± 1)</td>
<td>(19 ± 5)</td>
<td>(19 ± 5)</td>
<td>(15 ± 2)</td>
</tr>
<tr>
<td>MC microsomes</td>
<td>26,190</td>
<td>8,440</td>
<td>15,500</td>
<td>14,840</td>
<td>3,710</td>
</tr>
<tr>
<td>17 μg/ml</td>
<td>(27.9)</td>
<td>(9.0)</td>
<td>(16.5)</td>
<td>(15.8)</td>
<td>(4.0)</td>
</tr>
<tr>
<td>N.D.³³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean and standard deviation of four preparations (CO, MC) and six preparations (PB).

β Single metabolite as a per cent of the total metabolites formed in an incubation, mean and standard deviation.

³ Mean of duplicate determinations.

³³ 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 Jernström 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 incubations (Table I) so that cytochrome P-450 between nuclear envelope and microsomes after 3-methylcholanthrene induction. The specific activity for benzo(a)pyrene metabolism per unit cytochrome P-450 was the same in both microsomes and 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 $\lambda_{\text{max}}$ 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 $\lambda_{\text{max}}$ 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.
forms have been quantitated by high pressure liquid chromatography via their tetrod hydrolysis and triol reduction products (3, 30) (Fig. 1) using enzymatically formed BP 7,8-dihydrodiol as the substrate (Table IV). The ratio of tetrods formed by hydrolysis of pure enantiomers and the relative retention times were similar to those reported by Young et al. (7). These 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 with 3-methylcholanthrene induction which were comparable to oxygenation rates.

Fraction of liver microsomes by means of sodium decyl sulfate-polyacrylamide gel electrophoresis has indicated the presence of many different forms of cytochrome P-450 (32, 33). However, the nuclear membrane contains insufficient cytochrome P-450 to use this means of identifying the cytochrome.

The binding of ligands to the substrate site of a P-450 cytochrome induces a type I spectral response. Liver microsomal P-450 cytochromes have broad specificity but each can be characterized by the affinities of a set of ligands for the type I site of that particular cytochrome. SKF 525A, camphor, and lidocaine have been shown to differentiate between various forms of cytochrome P-450 in rat liver microsomes (34). Several forms bind each ligand, but we have found a high affinity site (Kₐ) is 10 times lower than for other sites) for each ligand in phenobarbital-induced rat liver microsomes (35). Two other forms were distinguished in the membrane by weak interactions with, respectively, camphor and lidocaine, while a sixth form was distinguished by binding testosterone and not the other ligands (35).

The type I spectral response produced by saturation of the binding site 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. Powsis 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

\[ \text{Enzyme source} \quad \text{BP 4,5-oxide hydration} \]

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>BP 4,5-oxide hydration nmoles/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.32 ± 0.41*</td>
</tr>
<tr>
<td>MC</td>
<td>3.97 ± 0.21*</td>
</tr>
<tr>
<td>PB</td>
<td>3.64 ± 0.33*</td>
</tr>
<tr>
<td>Microsomes</td>
<td>10.34 ± 0.52</td>
</tr>
<tr>
<td>Control</td>
<td>11.61 ± 0.94</td>
</tr>
<tr>
<td>MC</td>
<td>29.93 ± 3.34</td>
</tr>
</tbody>
</table>

\[ a \text{ Mean and standard deviation of at least four determinations.} \]

\[ b \text{ Significantly different from control, } p < 0.01. \]
Nuclear Envelope Mixed Function Oxidase System

Metabolism of [3H]BP 7,8-dihydrol by rat liver nuclear envelope and microsomal membrane

Enzymatically formed [3H]BP 7,8-dihydrol was incubated in 0.5-ml mixtures for either 4 min (MC nuclear membrane, 10 μM substrate) or 15 min as described under "Experimental Procedures." The amount of protein in each incubation was as follows: nuclear membrane-control, 2.3 mg; MC-10 μM, 2.3 mg; MC-40 μM, 1 mg; microsomes-control, 1 mg, MC, 0.3 mg. Metabolite fractions are quantitated from the amount of radioactivity found to elute with the same retention time as the standards illustrated in Fig. 1. The amount of radioactivity extractable after a 15-min incubation ranged from 99.5% (control nuclear envelope) to 92.7% (MC-microsomes, 40 μM).

Exhibit type I spectral interactions. Thus, after induction by 3-methylcholanthrene, a substantial proportion of cytochrome P-450 in the liver nuclear envelope clearly shows 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 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, = 3.6 μM) and low affinity sites.
affinity ($K_d = 35 \mu M$) sites in phenobarbital-induced liver microsomes but to only one site ($K_d = 6.1 \mu M$) 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, however. 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 vigorous criterion establishing the absence of contamination by endoplasmic reticulum is based upon the occurrence of an endogenous protein kinase in the endoplasmic reticulum that phosphorylates intrinsic microsomal membrane proteins not found in the nuclear envelope. Incubation of highly purified nuclear envelope under conditions optimal for phosphorylation yields no 32P-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 nmol 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 same as those found in the endoplasmic reticulum.
same in both membranes, irrespective of inducer but that, of course, these forms differ with the mode of induction. These experiments suggest, therefore, that most of the nuclear envelope cytochrome P-450 found in control and phenobarbital-treated animals cannot function in benzo(a)pyrene metabolism. The significant increase in phenols at the expense of dihydriodiol metabolites, and changes in the relative proportions of the dihydriodiol, clearly derive directly from the lower levels of epoxide hydratase in nuclear envelope preparations and can be mimicked by decreasing the concentration of protein in microsomal incubations. When epoxide hydratase is decreased, hydration decreases relative to the nonenzymatic phenolic rearrangement. As would be expected, the least stable BP 9,10-oxide is most affected by this change. When cytochrome P-450 and epoxide hydratase levels were comparable in 3-methylcholanthrene-induced nuclear envelope and microsomal incubations, 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 $K_p$ 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 membrane 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 ($K_p < 1 \mu M$) 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. 25 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-dihydriodiol 9,10-oxides 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-dihydriodiol 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-dihydriodiol in the nuclear envelope, the (-)trans-7,8-dihydriodiol was prepared enzymatically by the stereospecific oxygenation of benzo(a)pyrene, as has previously been described (7). Oxygenation at the 9,10 position of BP 7,8-dihydriodiol leading to formation of the anti isomer 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-dihydriodiol, 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-dihydriodiol 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-dihydriodiol in the nuclear membrane 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 c 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.

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enzymes is specifically associated with expression of genes at the Ah locus. 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 may contribute significantly to cell transformation during long term exposure to these compounds.

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