Genetic Regulation of Aryl Hydrocarbon Hydroxylase Induction

V. SPECIFIC CHANGES IN SPIN STATE OF CYTOCHROME P₄₅₀ FROM GENETICALLY RESPONSIVE ANIMALS*

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SUMMARY

The genetically controlled expression of hepatic aryl hydrocarbon (benzo(a)pyrene) hydroxylase induction in inbred and hybrid mice by aromatic hydrocarbons in vivo is associated, in freshly prepared oxidized microsomes, with increases in high spin cytochrome P₄₅₀ having a set of electron paramagnetic resonance signals at g = 8.0, 3.7, and 1.7. Phenobarbital causes similar rises (2- to 3-fold) in both high (g = 8.0, 3.7, and 1.7) and low spin (g = 2.4, 2.3, and 1.9) hepatic P₄₅₀ from any of the inbred or hybrid mice examined. Acetone treatment of microsomes in vitro destroys the g = 8.0, 3.7, 1.7 signals without affecting the hydroxylase activity, whereas Triton X-100 or sodium dodecyl sulfate affects the enzyme activity more so than the high spin signals. Substrate binding produces interconversion between high and low spin P₄₅₀. In vitro treatment of microsomes with sodium dodecyl sulfate or sodium deoxycholate or at 55°C for 5 min causes the irreversible loss of high and low spin P₄₅₀. Induction of the hydroxylase activity is associated with the increased g = 8.0, 3.7, and 1.7 signal heights in the kidney from 3-methylcholanthrene-treated rabbit and in the liver or kidney from 3-methylcholanthrene-treated rat, but not in liver from aromatic hydrocarbon-treated rabbit. Hence, aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons and the increase in high-spin cytochrome P₄₅₀ are not always necessarily related.

These results indicate that aromatic hydrocarbon treatment of the genetically responsive animal, rather than treatment of any animal with aromatic hydrocarbons per se, causes an increase in a P₄₅₀ species which has iron in a preferred high spin configuration. This preferred spin state may reflect a change near the P₄₅₀ active site, such as the presence of new apoenzyme(s) or a different configuration. There is another high spin protein iron having a g = 5.95 signal; this most likely represents a denatured form of P₄₅₀.

Membrane-bound mono-oxygenases (5, 6) are multicomponent enzyme systems (7–10) which require NADPH and molecular oxygen for the oxidative metabolism of drugs, insecticides, and polycyclic hydrocarbons, as well as numerous lipophilic endogenous compounds. Any fluctuation in these enzyme activities may therefore influence the intensity and duration of drug action and also the rate of metabolism of insecticides, chemical carcinogens, and a wide variety of normal body substrates (7). Mono-oxygenases have as an active site for their oxidative function cytochrome P₄₅₀, so named (11) because the reduced form of this hemoprotein upon combination with CO usually has a Soret maximum at about 450 nm. This microsomal CO-binding pigment comprises a mixture of at least two distinguishable forms (2–4, 10, 12–34). A spectrally distinct species of P₄₅₀ is associated with polycyclic hydrocarbon treatment of the intact animal (2–4, 10, 12, 13, 15, 16, 19, 25–32) or cell cultures (22), whereas the binding of drugs or steroids to mammalian P₄₅₀ (14, 17, 18, 20, 21, 23, 24) or bacterial P₄₅₀ (32–34) alters the spin state of the iron in the hemoprotein. The relationship between the spectrally distinct form of P₄₅₀ appearing in polycyclic hydrocarbon-treated animals and the changes in spin state produced by the binding of endogenous or exogenous substrates to the pigment has not been resolved. As will be shown in this report, some of the confusion has arisen from the use of detergent-treated submicrosomal particles (14–16, 25), and from samples stored frozen for some time (27, 32).

Studies from this laboratory have been directed at understanding the subcellular events which regulate the induction and de

* The previous papers of this series have appeared (1–4). Portions of this work were presented at the Meeting of the Federation of the American Society for Experimental Biologists, Atlantic City, New Jersey, April, 1972, and at the Second International Symposium on Microsomes and Drug Oxidations, Stanford, California, July, 1972.
exists.

near the Ah locus and that a third allele at the same locus probably have evidence (in preparation) that the induction of other mono-

strain for the Ah" allele, and the DBA/S strain (or D2) is arbi-

tarly chosen as the prototype strain for the Ahd allele. There-

C57BL/6 strain (or 136) is arbitrarily chosen as the cross between C57BL/6 and DBA/S, NZW/BLN, or NZB/BLN locus

carbon hydroxylase induction by aromatic hydrocarbons that t,he

3-methylcholanthrene; EPR, electron paramagnetic resodaneef

t)he hydroxylase induction, 1,~ the same extent as that found in the

The allele Ah" will represent, the dominant gene, i.e. expression of

rate of breakdown. Since this enzyme may be a multicom-

from pre-existing moieties, or in the rate of both, compared with

of de nova synthesis or in the rate of activation of enzyme activity

pre-existing mieties, or in the rate of both, compared with

enzyme de nova synthesis, and activation can be described only

after an induction-specific protein is characterized. Thus, the

rate of enzyme induction is used here only to express the rate at

which induced hydroxylase activity is accumulating.

The abbreviations used are: PB, sodium phenobarbital; MC, 3-

methylcholanthrene; EPR, electron paramagnetic resonance;

and SKF-525A, 2-diethylaminoethyl-2,2-diphenyIvalerate HCI.

Genetic Nomenclature for Mice (43), we propose for aryl hydro-

carbon hydroxylase activity, one of

several mixed-function oxygenases inducible by both aromatic hydrocarbons and PB (2, 4, 35, 40). As reported in this series

(1-4), the hydroxylase induction by polycyclic hydrocarbons occurs in certain strains of inbred and randommice but does not

occur in other inbred strains. Induction of the enzyme activity

in several tissues by various aromatic hydrocarbons segre-

gates as a single autosomal dominant gene (2, 3, 39-42), which

we suggest be designated the ab locus.8 On the other hand, the

extent of hepatic hydroxylase induction by PB is about the same

among more than 10 inbred strains of mice (Reference 2 and un-
published data). The genetically nonresponsive, or alah, mouse

thus is extremely useful as an important negative control: al-

though binding of aromatic hydrocarbons to the P450 active site and metabolism of these inducers undoubtedly take place (1, 2), specific phenomena related to the induction of hydroxylase ac-

tivity by aromatic hydrocarbons do not occur. In this report

we examine, with the use of EPR spectroscopy below 10° K, specific changes in the spin state of P450 in genetically responsive

and genetically nonresponsive animals treated with aromatic hydrocarbons or PB.

MATERIALS

MC was purchased from J. T. Baker Chemical Company

(Phillipsburg, New Jersey); benzo[a]pyrene, NADPH, NADH,

and camphor from Sigma (St. Louis, Missouri); o- and p-naphtho-

flavone from Aldrich Chemical Company (Milwaukee, Wiscon-

sin); PB from Merck and Company, Inc. (Rahway, New Jersey);

liquid hemol from Gardner Cynogenic Division (Pennsylvania);

Triton X-100 from New England Nuclear (Boston, Mass.);

Lubrol from I. C. I. American, Inc. (Stamford, Connecticut); and

sodium deoxycholate from LaPine Scientific Company

(Norwood, New Jersey). The remainder of compounds used in this

study were generous gifts: metyrapone (2-methyl-1, 2, 3, 3-

1-triy1-1-propanone) from Dr. J. J. Chait, Ciba Pharmaceutical

Company; 2-(4-chlorophenyl)benzothiazole from Dr. J. L. Leong,

Phillipsburg, New Jersey); benzo[a]pyrene, NADPH, NADH,

and genetically nonresponsive animals treated with aromatic hydro-

carbons or PB.

METHODS

Treatment of Animals—The animals were kept for breeding in

standard hardwood (beech, birch, and maple) sawdust bedding

in plastic cases and fed normal laboratory chow (Ralston Purina

Company, St. Louis, Missouri). We attempted to control as

completely as possible the animal room environment, i.e. an

automatic day-night (16 hours to 8 hours) cycle and avoidance

of exposure to pharmacologically active compounds such as
cigarette smoke and insecticides. Rabbits, rats, and mice were

held for a minimum of 1 week in this environment before any

experimental manipulations were performed. At the time of

experiments, mice of either sex (2) between 4 and 10 weeks of

age and sexually immature male rabbits or rats were always used.

Unless otherwise stated, the animals received one intraperitoneal

dose of MC or p-naphthoflavone (80 mg per kg of body weight)

in corn oil 24 hours before sacrifice; controls received corn oil

only. PB-treated mice received intraperitoneally 80 mg of PB

in 0.90% NaCl solution per kg of body weight on each of 3

successive days before sacrifice. Larger or smaller doses or a

greater number of doses of MC, p-naphthoflavone, or PB pro-

duced the same or lesser magnitudes of hydroxylase induction

All experiments were begun at the same hour of day. Liver or

kidney microsomal samples were prepared in 0.25 m potassium

phosphate buffer-30% glycerol, pH 7.25, exactly as previously

described (2, 3), except that suspensions ranged between 13 and

88 mg of protein per ml. Mouse liver microsomes were either

prepared from individual animals or combined from 3 mice and

treated as one sample. Approximately 0.5 ml of each sample

was added to an EPR quartz tube (Varian and Associates, Palo

Alto, California), creating a column (0.3 x 6.5 cm). The

remainder of each sample was diluted 10-fold for concomitant

determinations of hydroxylase activity and protein content (1-4,

39). The paramagnetic measurements of these suspensions were

linear between 13 and 88 mg of protein per ml. Unless other-

wise noted, the freshly prepared microsomal samples were kept

at 0-5° for 0 to 2 hours, until which time they were frozen in

liquid nitrogen and then examined at 9.6° K.

EPR Spectroscopy—All EPR measurements were carried out at

9.6° K with the use of a conventional X-band spectrometer with

100 KHz field modulation. The low temperature was main-

tained by the evaporation of liquid helium through a single-

jacketed variable temperature dewar and by the regulation of

flow rate by adjusting the current for a resistor in the helium

reservoir. The temperature of the sample was measured by a

calibrated germanium resistor (CR 1000, CryoCal, Incorporated,

Florida) immediately below the sample in the variable tempera-

ture dewar. During the measurement of any sample, fluctua-

tion of the temperature by this method varied less than 0.5°.

Throughout this study, preparation of each sample and EPR

measurements were performed always in the same manner; the
gain setting, the modulation amplitude, the klystron frequency

were always kept the same. In this report the signal intensities

are arbitrarily described in chart units per gain per mg of micro-

somal protein.
**RESULTS**

Effects of Aromatic Hydrocarbons or PB on EPR Signals in Mouse Liver Microsomes—Fig. 1 shows EPR absorption derivative spectra of hepatic microsomes from control C57BL/6N, MC-treated DBA/2N, and MC-treated C57BL/6N mice. The spectrum from control DBA/2N mice (not shown) was very similar to that from either the control C57BL/6N or the MC-treated DBA/2N strains. Readily distinguishable absorption features include effective g values near 8.0, 3.7, and 1.7 caused by a high spin hemoprotein having a strained rhombic configuration (27); the set of g values of about 2.4, 2.3, and 1.9 due to "microsomal Fe₅₅₅₅," or low spin P₄₅₀ (44); a g = 4.4 signal presumably representing mononuclear ferric iron in an almost completely rhombic or tetrahedral environment (27, 45); and signals having approximate g values of 6.0 and 3.2. Features at g = 3.2 may reflect the lowest field signal of the g tensor for microsomal cytochrome b₅ low spin iron (46). In vivo MC treatment of the C57BL/6N mouse, but not that of the DBA/2N mouse, produced a 3- to 4-fold increase in the set of EPR signals related to high spin P₄₅₀ (g = 8.0, 3.7, and 1.7). Two hours after the intraperitoneal administration of MC to C57BL/6N mice, however, at a time when the liver contains maximal levels of the polycyclic hydrocarbon, we found no increases in the g = 8.0, 3.7, and 1.7 signals or in specific hydroxylase activity. The set of g = 8.0, 3.7, and 1.7 signals was not found at temperatures of the sample greater than 10³ K.

For 20 consecutive determinations, we compared the relative differences among each of the g = 8.0, 3.7, and 1.7 signal heights and found a correlation coefficient r of 0.96, indicating that these three signals represent the three principal values of the g tensor for the microsomal hemoprotein (p < 0.001). This relationship is in close agreement with the work of Peisach and Blumberg (27) who gave their observed and theoretical g values for the high spin form of P₄₅₀. There were no other striking changes (Fig. 1) among the remaining EPR signals after MC treatment of C57BL/6N mice.

We found that contributions by one species of iron to the total EPR absorbance derivative spectrum were not only difficult to determine but gave rise to inaccuracies, because of variable amounts of a broad underlying signal between g = 2.6 and 1.8 and because of varying amounts of a g = 2.0 signal attributable to free radicals. Therefore, rather than attempting to determine the integrated area under any EPR signals, we chose instead to examine the heights of each individual signal known to represent a different form of microsomal iron, i.e., g = 8.0, g = 5.95, g = 4.44, and g = 2.27. Comparison of the peak height among signals representing any one paramagnetic species is valid, because the peak height is proportional to the total intensity. However, direct comparison of any signal from one paramagnetic species with any signal reflecting a different paramagnetic species may be invalid for various reasons. For example, at the microwave power level and the temperature used in the present study, the g = 2.4, 2.3, 1.9 set of signals due to the low-spin hemoprotein iron was found to be approximately 50% saturated. On the other hand, the g = 8.0, 3.7, 1.7 set of signals attributable to high spin iron was not saturated; yet, the observed signal intensity does not represent the total amount of the high spin species, since the signal is derived only from the molecules which are thermally populated in the lowest Kramer's doublet (S° = 1/2) of the high spin d⁵ iron.

Table I shows the relationship between changes in EPR signal intensities and in vivo treatment of mice with MC or PB. A total of 79 determinations is included. The control mouse and the genetically nonresponsive MC-treated mouse showed no statistically significant differences and therefore are combined as one group. MC treatment only of genetically responsive strains caused a 3.3-fold (p < 0.001) increase in P₄₅₀ having the g = 8.0 signal, a 30% Rise (p < 0.01) in the g = 3.35 EPR signal, a 10% increase (p = 0.04) in P₄₅₀ having the g = 2.27 signal, and a 3.9-fold rise in hydroxylase activity. The g = 4.44 signal was not significantly (p > 0.10) changed. Hence, the likelihood of the parent compound or a metabolite affecting the spin state of hepatic microsomal P₄₅₀ after in vivo treatment of an animal with an aromatic hydrocarbon (27) is not necessarily the cause.

Fig. 1. EPR absorption derivative spectra of liver microsomes from control C57BL/6N (top), MC-treated DBA/2N (middle), and MC-treated C57BL/6N (bottom) mice. Protein concentrations were 38.8, 31.9, and 35.8 mg per ml for the control, MC-treated DBA, and MC-treated DBA mice, respectively, and specific hydroxylase activities were 500, 540, and 1960, respectively. The g values and field strength (H, in gauss) are indicated for various important features of the spectra. The spectrometer gain and the temperature (9.6 K) of the sample were kept constant for each of these spectra.

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TABLE I

Effects of MC or PB in vivo on EPR signals and aryl hydrocarbon hydroxylase activity in mouse liver microsomes

The values of EPR signal height and of specific hydroxylase activity are given as the mean ± standard deviation. The MC-treated genetically nonresponsive and control mice are combined together as one group, because no significant differences were found. These determinations are based on 17 control, 18 MC-treated genetically nonresponsive, 34 MC-treated genetically responsive, and 10 PB-treated samples. Ten inbred or randomly bred strains of mice are included. In this table and in subsequent figures and tables, the signal height for one sample can be validly compared only with the corresponding signal height for other samples (see text). We found that the g = 3.2 signal (Fig. 1) remained small and that changes in the intensity of this signal were not consistently related to any of the other signals.

<table>
<thead>
<tr>
<th>Signal</th>
<th>Control or MC-treated genetically nonresponsive</th>
<th>MC-treated genetically responsive</th>
<th>PB-treated</th>
<th>Magnitude of increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>g = 8.01</td>
<td>± 0.0012</td>
<td>0.0040</td>
<td>± 0.0031</td>
<td>2.6</td>
</tr>
<tr>
<td>g = 5.95</td>
<td>± 0.0029</td>
<td>± 0.0011</td>
<td>± 0.0068</td>
<td>1.4</td>
</tr>
<tr>
<td>g = 4.44</td>
<td>± 0.0017</td>
<td>± 0.0024</td>
<td>± 0.0050</td>
<td>0.92</td>
</tr>
<tr>
<td>g = 2.27</td>
<td>± 0.0013</td>
<td>± 0.0003</td>
<td>± 0.0008</td>
<td>0.0010</td>
</tr>
<tr>
<td>Specific hydroxylase activity</td>
<td>± 0.0062</td>
<td>± 0.0009</td>
<td>± 0.0015</td>
<td>1.1</td>
</tr>
<tr>
<td>Specific hydroxylase activity</td>
<td>± 0.0015</td>
<td>± 0.0016</td>
<td>± 0.0030</td>
<td>0.98</td>
</tr>
<tr>
<td>Specific hydroxylase activity</td>
<td>± 480</td>
<td>± 1870</td>
<td>± 510</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Signal heights are expressed in chart units per gain per mg of microsomal protein.

PB treatment of AhAh or ahah mice produced a 2.8-fold increase in g = 8.01 P450, a 50% rise (p < 0.01) in the g = 5.95 signal, a 3.1-fold magnitude of increase in g = 2.27 P450, and a 2.6-fold stimulation of specific hydroxylase activity. We examined the EPR signals of g = 2.45, 2.27, and g = 1.94 with respect to rates of change and calculated a correlation coefficient r of 0.96, indicating that these three signals are related to (p < 0.001) the three principal values of the g tensor for low spin P450 (44).

We have previously shown (3) that MC and PB are additive as inducers in the genetically responsive animal but that only PB is capable of inducing the hydroxylase activity in the genetically nonresponsive mouse. Fig. 2 illustrates changes in the four distinct EPR signals from C57BL/6N and DBA/2N mice treated with either MC alone or MC plus PB. Similar results were obtained with the C3H/HeN strain in which the enzyme is inducible by MC and with the AKR/N strain in which hepatic hydroxylase activity is not inducible by MC. As predicted, in the DBA/2N mouse treated with MC plus PB, the g = 8.01 signal was increased to a level intermediate between that observed in the MC-treated DBA/2N mouse and that seen in the MC-treated C57BL/6N mouse. And, in the C57BL/6N mouse treated with MC plus PB, the inducer are additive in their effect on increasing the g = 8.01 signal. This relationship was not seen for the other high spin signals (g = 5.95 and 4.44). In animals treated with MC plus PB, the low spin P450 (g = 2.27) was considerably more increased in the C57BL/6N mouse than that in the DBA/2N strain.

In spite of the problems in estimating the total content of high and low spin P450 that we have already discussed, Table II shows such an approximation by means of doubly integrating “ideal” EPR derivative spectra of microsomes from control, MC-, or PB-treated mice. We realize our estimations may be in error by 10% or even more, but the relative amounts are worth noting. Hence, we found that the total P450 content in control or MC-treated genetically nonresponsive mice and in PB-treated mice divided approximately equally; one-half low spin and one-half high spin, whereas about four-fifths of the cytochrome iron in MC treated genetically responsive mouse was in the high spin form. The total P450 content was increased about 2.8-fold in PB-treated mice and about 2.4-fold in MC-treated genetically responsive mice. These magnitudes of increase are in remarkably good agreement with our spectrophotometric determinations (11) of P450 from these same mice (Reference 2 and unpublished data).

From the data in Figs. 1 and 2 and Tables I and II, therefore, the rise in g = 8.01 P450 but not low spin P450 in response to MC is somehow mediated at the genetic level, whereas the PB-caused increases in both g = 8.01 and 2.27 P450 are not controlled by the same genetic event. Moreover, the magnitude of increase in induced hydroxylase activity more closely parallels that of the g = 8.01 EPR signal than that of low spin P450 or any other signal representing paramagnetic iron.

Effects of Acetone, Detergents, and Freezing on EPR Signals in Mouse Liver Microsomes—Table III shows that acetone or detergent treatment of microsomes in vitro causes a lack of correlation between the g = 8.01 signal and either control or induced hydroxylase activity. A final concentration of 0.19 M acetone had essentially no effect on the specific hydroxylase activity while destroying most of the g = 8.01 signal. Alternatively, Triton X-100 did not decrease the g = 8.01 signal, whereas...
TABLE II

Estimated content of hepatic microsomal high and low spin cytochrome P₄₅₀ from control, MC-treated, and PB-treated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control or MC-treated genetically nonresponsive</th>
<th>MC-treated genetically responsive</th>
<th>P450-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>High spin P₄₅₀ content</td>
<td>38,000</td>
<td>125,000</td>
<td>98,000</td>
</tr>
<tr>
<td>Per cent of total</td>
<td>50%</td>
<td>81%</td>
<td>54%</td>
</tr>
<tr>
<td>Low spin P₄₅₀ content</td>
<td>36,000</td>
<td>20,000</td>
<td>82,000</td>
</tr>
<tr>
<td>Per cent of total</td>
<td>41%</td>
<td>19%</td>
<td>46%</td>
</tr>
<tr>
<td>Total P₄₅₀ content</td>
<td>64,000</td>
<td>154,000</td>
<td>180,000</td>
</tr>
</tbody>
</table>

* Expressed as chart units² per gram per mg of microsomal protein.

Examples (see "Discussion").

by some genetic factor—rather than by treatment of animals with polycyclic hydrocarbons per se (27, 32).

TABLE III

Effects of acetone or various detergents in vitro and of storage at −10°C on EPR signals and aryl hydrocarbon hydroxylase activity on liver microsomes from control or MC-treated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>g = 8.01</th>
<th>g = 5.95</th>
<th>g = 4.44</th>
<th>g = 2.27</th>
<th>Specific hydroxylase activity</th>
<th>Hydroxylase activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-treated D2...</td>
<td>0.0010</td>
<td>0.0021</td>
<td>0.0015</td>
<td>0.0005</td>
<td>510</td>
<td>98%</td>
</tr>
<tr>
<td>+ Acetone...</td>
<td>0.0002</td>
<td>0.0018</td>
<td>0.0013</td>
<td>0.0052</td>
<td>500</td>
<td>98%</td>
</tr>
<tr>
<td>+ Triton X-100...</td>
<td>0.0014</td>
<td>0.0023</td>
<td>0.0014</td>
<td>0.0060</td>
<td>85</td>
<td>16%</td>
</tr>
<tr>
<td>+ Sodium dodecyl sulfate...</td>
<td>0.0010</td>
<td>0.0031</td>
<td>0.0011</td>
<td>0.0030</td>
<td>0.40</td>
<td>0%</td>
</tr>
<tr>
<td>+ Lubrol...</td>
<td>0.0007</td>
<td>0.0015</td>
<td>0.0014</td>
<td>0.0054</td>
<td>6.4</td>
<td>1.2%</td>
</tr>
<tr>
<td>+ Sodium deoxycholate...</td>
<td>0.0007</td>
<td>0.0043</td>
<td>0.0012</td>
<td>0.0031</td>
<td>82</td>
<td>16%</td>
</tr>
<tr>
<td>Storage at −10°C for 2 days</td>
<td>0.0006</td>
<td>0.0030</td>
<td>0.0018</td>
<td>0.0039</td>
<td>140</td>
<td>27%</td>
</tr>
<tr>
<td>MC-treated B6...</td>
<td>0.0040</td>
<td>0.0014</td>
<td>0.0015</td>
<td>0.0055</td>
<td>1860</td>
<td>96%</td>
</tr>
<tr>
<td>+ Acetone...</td>
<td>&lt;0.0001</td>
<td>0.0018</td>
<td>0.0020</td>
<td>0.0039</td>
<td>1.89</td>
<td>100%</td>
</tr>
<tr>
<td>+ Triton X-100...</td>
<td>0.0040</td>
<td>0.0005</td>
<td>0.0018</td>
<td>0.0028</td>
<td>100</td>
<td>10%</td>
</tr>
<tr>
<td>+ Sodium dodecyl sulfate...</td>
<td>0.0064</td>
<td>0.0028</td>
<td>0.0016</td>
<td>0.0036</td>
<td>&lt;0.02</td>
<td>0%</td>
</tr>
<tr>
<td>+ Lubrol...</td>
<td>0.0060</td>
<td>0.0010</td>
<td>0.0012</td>
<td>0.0031</td>
<td>23</td>
<td>1.3%</td>
</tr>
<tr>
<td>+ Sodium deoxycholate...</td>
<td>0.0030</td>
<td>0.0052</td>
<td>0.0024</td>
<td>0.0037</td>
<td>580</td>
<td>31%</td>
</tr>
<tr>
<td>Storage at −10°C for 2 days</td>
<td>0.0033</td>
<td>0.0016</td>
<td>0.0012</td>
<td>0.0046</td>
<td>1830</td>
<td>98%</td>
</tr>
</tbody>
</table>

* Units per mg of microsomal protein.

The microsomal pellet, prepared as usual, was then frozen and stored at −10°C for 2 days. Following storage, the microsomal suspension was prepared in the usual manner and examined for paramagnetic changes and specific hydroxylase activity.

by some genetic factor—rather than by treatment of animals with polycyclic hydrocarbons per se (27, 32).

Table IV shows the effects of temperature changes on the EPR signals from the same sample of either control microsomes or microsomes from MC-treated C57BL/6N mice. Whereas thawing the samples to 0°C for 5 min had no effect on the EPR spectra, a second thawing from 9.6 K to 37°C for 5 min enhanced the g = 8.01 signal, yet the EPR signals representing other species of iron did not change appreciably. A third thawing from 9.6 K to 55°C for 5 min produced significant increases in the g = 5.95 signal. This inverse relationship may be related to the observation of Murakami and Mason (47), whereby denaturation of low spin P₄₅₀ by p-chloromercuriphenylsulfonate in vitro resulted in the concomitant appearance of a high-spin hemoprotein with an EPR absorption near g = 6. The g = 4.44 signal (Table IV) remained remarkably constant throughout all of the temperature changes.
specificity similar to that for the polycyclic hydrocarbons in eliciting the enzyme induction only in AhAh or Ahah mice (3), activity (2, 3), and that the increase did not occur in the inbred randombred National Institutes of Health General Purpose DBA/ZN, AKR/N, NZB/BLN, or NZW/BLN strain, all of which contain MC-inducible hydroxylase inbred C57BL/6N, C3II/HeN, and BALB/cAnN strains and the which do not have the hepatic hydroxylase inducible by MC (2, 3). Moreover, \(\beta\)-naphthoflavone, an inducer which has a substantial rises in the \(g = 8.01\) signal. An exception was SKF-525A, a type I compound which behaved similarly to \(I-(2\text{-isopropylphenyl})\)imidazole and metyrapone. However, SKF-525A possesses a nitrogen atom and therefore an unoccupied propylphenyl)imidazole and metyrapone. Thus, in microsomes from MC-treated C57BL/6N mice, MC and \(\alpha\)-naphthoflavone, type I compounds (4), generally produced increases in the \(g = 8.01\) signal and decreases in the \(g = 2.27\) signal. Most likely, a more marked effect could have been observed with higher concentrations of MC or \(\alpha\)-naphthoflavone, but this experiment was not performed in greater detail. In microsomes from both MC-treated C57BL/6N mice and either control or MC treated DBA/2N mice, the type II compounds 1-(2-isopropylphenyl)imidazole and metyrapone caused marked decreases in the \(g = 8.01\) signal and substantial rises in the \(g = 2.27\) signal. An exception was SKF-525A, a type I compound which behaved similarly to 1-(2-isopropylphenyl)imidazole and metyrapone. However, SKF-525A possesses a nitrogen atom and therefore an unoccupied pair of electrons, the necessary requirement for compounds to cause Type II binding spectra (51). No significant change from one spin state of \(P_{450}\) to the other was found with 2-(4'-chlorophenyl)benzothiazole and DL-camphor at the concentrations used.


\[
\begin{array}{|c|c|c|c|}
\hline
\text{Compound} & \text{\(g = 8.01\)} & \text{\(g = 5.95\)} & \text{\(g = 2.27\)} \\
\hline
\text{Control} & 0.0010 & 0.0016 & 0.0010 & 0.0055 \\
\text{MC} & 0.0007 & 0.0018 & 0.0008 & 0.0050 \\
\text{\(\alpha\)-Naphthoflavone} & 0.0010 & 0.0022 & 0.0011 & 0.0052 \\
\text{1-(2-Isopropylphenyl)imidazole} & <0.0001 & 0.0008 & 0.0008 & 0.0006 \\
\text{Metyrapone} & 0.0003 & 0.0018 & 0.0011 & 0.0055 \\
\text{SKF-525A} & 0.0010 & 0.0020 & 0.0009 & 0.0055 \\
\text{DL-Camphor} & 0.0008 & 0.0007 & 0.0014 & 0.0074 \\
\hline
\end{array}
\]

**Fig. 3.** Effects of MC (left) or \(\beta\)-naphthoflavone (BNF, right) on the \(g = 8.01\) signal intensity in liver microsomes from various strains of mice. Each hatched bar represents the control value and the solid bar immediately beside the control bar depicts the signal height after MC or \(\beta\)-naphthoflavone treatment of the mice 24 hours before assay. The magnitude of hydroxylase induction (i.e., the induced-control ratio) for each of the 12 sets of values from left to right was 3.5, 3.2, 2.9, 3.0, 0.92, 1.0, 0.71, 0.92, 6.5, 0.87, 3.9, and 0.93.

caused significant increases in the \(g = 8.01\) signal only in genetically responsive inbred strains of mice.

Fig. 4A illustrates that the increase in the \(g = 8.01\) signal segregates as a single autosomal dominant gene. Thus, whereas MC treatment of the F1 population or offspring from the B6D2 F1 × B6 backcross always produced the full 3- to 4-fold rise in the

\[\text{V. Ullrich, personal communication.}\]
high-spin signal intensity, MC treatment of offspring from the B6D2 F₁ × D2 backcross and of the F₁ generation elicited bimodal populations wherein approximately one-half and one-fourth, respectively, of the offspring were genetically nonsensitive to the aromatic hydrocarbon. The same distribution of genetic expression was found in offspring from various crosses between inbred and hybrid C57BL/6N and DBA/2N mice (not shown). Further, in Fig. 4B the specific hydroxylase activity (in units per mg of microsomal protein) as a function of the g = 8.01 signal height among MC-treated offspring from the D2 × B6D2 F₁ backcross. Correlation coefficient, r = 0.93 (p < 0.001).

**TABLE VI**

<table>
<thead>
<tr>
<th>Source and treatment</th>
<th>g = 8.01</th>
<th>g = 5.93</th>
<th>g = 4.44</th>
<th>g = 2.27</th>
<th>Specific Specific Specific Specific activity* units/mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.0036</td>
<td>0.0014</td>
<td>0.0007</td>
<td>0.0040</td>
<td></td>
</tr>
<tr>
<td>Control*</td>
<td>0.0008</td>
<td>0.0013</td>
<td>0.0008</td>
<td>0.0032</td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>0.0037</td>
<td>0.0022</td>
<td>0.0007</td>
<td>0.0057</td>
<td></td>
</tr>
<tr>
<td>MC*</td>
<td>0.0064</td>
<td>0.0011</td>
<td>0.0004</td>
<td>0.0035</td>
<td></td>
</tr>
<tr>
<td>L-Naphthoflavone*</td>
<td>0.0079</td>
<td>0.0006</td>
<td>0.0004</td>
<td>0.0033</td>
<td></td>
</tr>
<tr>
<td>Rat liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.0005</td>
<td>0.0015</td>
<td>0.0008</td>
<td>0.0068</td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>0.0010</td>
<td>0.0023</td>
<td>0.0009</td>
<td>0.0049</td>
<td></td>
</tr>
<tr>
<td>Rabbit kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.0001</td>
<td>0.0008</td>
<td>0.0002</td>
<td>0.0004</td>
<td>0.63</td>
</tr>
<tr>
<td>MC</td>
<td>0.0005</td>
<td>0.0010</td>
<td>0.0013</td>
<td>0.0012</td>
<td>0.15</td>
</tr>
<tr>
<td>Rat kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.0001</td>
<td>0.0008</td>
<td>0.0006</td>
<td>0.0004</td>
<td>2.3</td>
</tr>
<tr>
<td>MC</td>
<td>0.0003</td>
<td>0.0010</td>
<td>0.0013</td>
<td>0.0007</td>
<td>190</td>
</tr>
</tbody>
</table>

* Units per mg of microsomal protein.
* These three particular rabbits were treated with 1 dose of corn oil only, MC, or β-naphthoflavone 3 days prior to sacrifice.

48 Gauss, the half-width of the g = 8.01 signal in hepatic microsomes from the control or MC-treated rabbit or in kidney microsomes from rabbit or rat was not greater than 26 Gauss. This observation suggests to us that the high spin P₄₅₀ iron responsible for the g = 8.01 signal is more specific, or homogeneous, in rabbit liver and kidney and in rat kidney, compared with that in rat or mouse liver. It is therefore possible that a MC-inducible mono-oxygenase activity other than ary hydrocarbon hydroxylase is associated with this narrow signal. To test this hypothesis we examined p-nitroanisole O-demethylase (52) and found that neither of these activities is induced by MC in rabbit liver. To date, therefore, no mixed-function oxidase induction in hepatic microsomes from MC-treated rabbit has been associated with the increased g = 8.01 signal.

**DISCUSSION**

We have found that the genetically controlled expression of microsomal hydroxylase induction in the mouse by aromatic hydrocarbons is associated with increases in high-spin P₄₅₀ having a set of EPR signals at g = 8.0, 3.7, and 1.7 in freshly prepared microsomes. Furthermore, the magnitude of increase in the hydroxylase activity and in the intensity of the g = 8.01 signal in rabbit kidney and rat liver or kidney indicates that these phenomena may be related in other species besides mice, and in kidney as well as in liver microsomes. However, the hydroxylase induction and the increased g = 8.01 signal height can be unrelated in certain instances, (a) acetone treatment of microsomes in vitro destroys the EPR signal without affecting the hydroxylase activity; (b) treatment of microsomes in vitro with Triton X-100 or sodium dodecyl sulfate more seriously affects the enzyme activity than the g = 8.01 signal; (c) substrate binding elicits inverse changes in the concentration of the g = 8.01 and g = 2.27 P₄₅₀ species; and (d) treatment of the rabbit with MC in...
We therefore conclude from these findings (Fig. 6) that MC treatment of a genetically responsive animal produces a P450 species having iron in a preferred high-spin configuration. MC treatment per se does not effect this change, as is the case in the MC- or β-naphthoflavone-treated ahah mouse, in which the aromatic hydrocarbon parent compound and metabolites presumably bind specifically and nonspecifically to the microsomal membrane. Hence, aromatic hydrocarbons having a certain structural specificity (3) interact at some subcellular site, thereby causing new RNA and protein synthesis (39), regulated in some manner by the dominant Ah allele. The induction-specific protein then interacts in some way either in the cytosol or in the membrane with newly synthesized heme or hemoprotein, or with the aromatic hydrocarbon substrate, or both, in such a manner that high-spin P450 with g values of 8.0, 3.7, and 1.7 is proportionately increased. It was previously suggested by Jefcoate and Gaylor (24) that a new species of P450 produced by MC may correspond to distinct membrane proteins or configurations and not simply to two forms of the same hemoprotein in an equilibrium by differences in such parameters as ionic strength or pH. We therefore suggest (Fig. 6) that, whatever the genetically regulated effect on increasing the high spin P450 is, this species possesses iron which can still be readily interconverted between the low and high spin forms by means of substrate binding (Table V) and metabolism of endogenous or exogenous compounds. This newly formed enzyme active site(s) may be caused by a new apoprotein and may display differences in response to inhibitors (4) or in metabolite formation. A thermal equilibrium between high and low spin iron in hemoproteins also may be important (32, 55, 56). For example, for the heme iron of methemoglobin, the thermal equilibrium between the high and low spin electronic configurations of the protein iron (57) is quite different from that of the other hemoproteins or iron complexes (58); the apoprotein may therefore determine the spin state. Our findings with EPR spectra from mouse, rabbit, or rat liver are not in accord with the observations of Peisach and co-workers (27, 32) who find no g = 8.0, 3.7, and 1.7 signals in microsomes from control or PB-treated rats or rabbits. We suspect this artifact is due to harmful freezing and thawing or other temperature changes in preparing the biological samples for EPR spectroscopy.

In vivo treatment of microsomes with sodium dodecyl sulfate or sodium deoxycholate or at 55°C for 5 min causes a rise in the g = 5.95 signal concomitant with a diminution in both the g = 8.01 and g = 2.27 signals. In fact, the increasing g = 5.95 signal is also associated with a lower field splitting of the signal. For example, PB-induced mono-oxygenase activities preferentially produce 3,4-epoxidation of bromobenzene (54) and ω and ω-1 hydroxylations of n-hexane, whereas the MC-induced enzyme system causes predominantly 2,3-epoxidation of bromobenzene (54) and ω-2 hydroxylation of n-hexane.

Another remote possibility for the lack of a g = 8.01 signal increase in ahah mice is that a unique metabolite of aromatic hydrocarbon produced only in the MC-treated genetically responsive animal binds to P450 in such a manner that the high-spin form of P450 is increased. This possibility was ruled out by mixing experiments with microsomes from both MC- or β-naphthoflavone-treated C57BL/6N and DBA/2N mice, wherein we found purely additive amounts of the g = 8.01 signal.
with a peak at about g = 6.6 (not shown). In reconstituting the hydroxylase activity from flavoprotein, lipid, and cytochrome fractions of liver from PB- and MC-treated rats, Lu and co-workers (59-61) observed a blue spectral shift in the Soret maximum of the reduced cytochrome CO complex (10), a typical type I spectrum with benzphetamine in vitro (59), preferential inhibition of the hydroxylase activity by α-naphthoflavone (60), and a g = 6 EPR signal without any detectable g = 8 signal (61). Their fractions are obtained (10, 59-61) with the use of sodium cholate. Likewise, Jefcoate and Gaylor (16) found a Soret maximum of the reduced homoprotein-CO complex at 448 nm and a g = 6.6 EPR signal in submicromolecular particles obtained from MC-treated rabbits by treatment of the particles with Lubrol in vitro. The g = 5.95 signal can also be converted to low spin P450 by type II substrates (Table V). However, in no instance were we able to regenerate the g = 8.01 signal from the g = 5.95 signal. Therefore (Fig. 6), we feel that the g = 5.95 signal reflects a partially denatured form of high spin microsomal hemoprotein. Can the g = 5.95 signal represent cytochrome P450 (11)? Actually, it has been shown that P450 produced by various mild treatments can be converted to P450 by polyols and reduced glutathione under appropriate conditions (62) and that mono-oxygenase activities can be partially restored (63) during such a conversion. We thus suggest that the g = 5.95 or 6.0 signal reflects either functional P450 (readily inter-changeable with P450 in the presence of sulfhydryl compounds such as glutathione or diithiothreitol) or nonfunctional P450, and that the remaining EPR peak or peaks of the hemoprotein iron are lost in the numerous signals of the g = 2.1 to 1.7 region.

With the use of n-octylamine binding to microsomes in vitro, we found (3) an increase in type a P450 and an apparent decrease in type b P450 associated with aromatic hydrocarbon treatment of the genetically responsive mouse. Yet, no difference in the extinction coefficients of the reduced hemoprotein-CO complexes from MC-treated dldh or ahah mice exists (3). In view of the EPR data presented here, we conclude that the extinction coefficients of high and low spin P450.

PB causes an increase in the g = 8.01 signal in ahah as well as AhAh mice. This finding indicates that the increased g = 8.01 signal intensity is probably not related per se to the genetic expression of hydroxylase induction by aromatic hydrocarbons. Or, if the two phenomena are intimately related, this would suggest that PB may derepress the ah allele or regulate in some manner the expression of aromatic hydrocarbon-inducible hydroxylyase activity. We have found (4) that several compounds which preferentially inhibit the MC-induced hydroxylase activity also block the enzyme activity from PB-treated mice more so than the control enzyme.

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REFERENCES

11. OMURA, T., AND SATO, R. (1964) J. Biol. Chem. 239, 2570-2578, 2379-2385
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Genetic Regulation of Aryl Hydrocarbon Hydroxylase Induction: V. SPECIFIC CHANGES IN SPIN STATE OF CYTOCHROME P450 FROM GENETICALLY RESPONSIVE ANIMALS
Daniel W. Nebert and Hideo Kon


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