The Effect of Benzpyrene, Phenobarbital, and Carbon Tetrachloride on Subcellular Metal Distribution and Microsomal Enzyme Activity

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SUMMARY

Alterations in the normal subcellular metal distribution of rat liver and lung have been produced by the acute administration of phenobarbital, carbon tetrachloride, and intratracheal 3,4-benzpyrene. Following the isolation of the various subcellular fractions by a standard technique, the metal composition of the subfractions was determined by atomic absorption spectrophotometry and expressed as 

metal per mg nitrogen.

Significant increases in lung microsomal copper and manganese and significant decreases in microsomal nickel and chromium were observed at 72 hr after intratracheal administration of 3,4-benzpyrene, at a dose which produced a 6-fold increase in lung 3,4-benzpyrene hydroxylase activity. The administration of phenobarbital (75 mg/kg) to rats for 3 days produced significant increases in liver microsomal copper, manganese, and zinc levels. In contrast, the administration of a subacute dose of carbon tetrachloride (0.5 ml/kg) produced a significant reduction of liver microsomal copper, manganese, and zinc, as well as a marked depression of liver aniline hydroxylase activity.

The subcellular metal changes observed in this study may reflect a redistribution of endogenous metal stores in the enzyme-responsive tissue, since these changes cannot be explained by alterations in the whole-organ metal content or the nitrogen content of the subfractions.

INTRODUCTION

The 1st systematic investigation of the normal metal content of subcellular fractions was made by Thiers and Vallee in 1957 (14). It was found at that time that certain metals are reproducibly distributed among the fractions of rat liver and that each fraction has a characteristic and significantly different pattern of metal concentration. In subsequent investigations by Wacker and Vallee (15), nucleic acid preparations were also found to contain significant normal concentrations of metals, including relatively high levels (60 to 100 ppm) of such previously undetected metals as nickel, chromium, and manganese.

The possible alteration of these normal metal levels in subcellular fractions and macromolecules by in vivo administration of foreign compounds has not been extensively investigated, probably because of the previous limitations of analytical instrumentation. With the advent of atomic absorption spectrophotometry, reliable measurements of such low concentrations of metals as are present in subcellular particles is now possible.

The studies of Dixon et al. (5) in our laboratory on the role of trace metals in chemical carcinogenesis, with reference to asbestos cancers, have shown that certain metals (present in asbestos and in tissue) affect the in vitro activity of the microsomal enzyme, BP2 hydroxylase (aryl hydrocarbon hydroxylase), in rat lung; specifically, copper activates and nickel and chromium inhibit the normal and induced activity of this enzyme. Although this study focused attention on the role of added metals in the control of BP metabolism, in vivo studies of the alteration of the normal subcellular metal distribution of these metals after administration of BP and of the effect of chronic metal administration on BP hydroxylase activity were not included.

This report presents the results of a study of the effect of the acute intratracheal administration of BP on subcellular metal distribution in rat lung. The actions of phenobarbital and carbon tetrachloride on liver subcellular metal distribution were also investigated. The primary aim of this study was to determine whether the induction or inhibition of microsomal enzyme activity in rat lung and liver is associated with subcellular trace metal changes. BP has been shown to be a potent in vivo and in vitro inducer of its own metabolism in pulmonary tissue by Wattenberg et al. (16); phenobarbital and carbon tetrachloride produce dramatic antagonistic effects on liver microsomal enzyme activity (4).

A preliminary screening of 11 elements after the various compounds used in this study indicated that the most profound changes occurred in 5 elements: copper, manganese, zinc, chromium, and nickel. Therefore, the present investigation was focused on these elements. The observed metal changes were correlated in time with alterations in microsomal enzyme activity in lung or liver.

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2 The abbreviation used is: BP, benzpyrene.
MATERIALS AND METHODS

Specific pathogen-free male Greenacres Laboratory\(^3\) (Cincinnati, Ohio) rats, weighing 210 to 240 g, were used in this work and were fed food and water ad libitum. BP was administered intratracheally as a single 8-mg dose in peanut oil solution. Phenobarbital was administered at a dose of 75 mg/kg/day i.p. for 3 days. Carbon tetrachloride was administered to 3 groups of rats in single doses of 0.25, 0.50, and 1.00 ml/kg p.o., respectively. The appropriate vehicle, water (1 ml/kg) or peanut oil (0.5 ml/kg), was administered to each set of control animals. No inflammatory response was evident in the lungs of control animals receiving peanut oil. The BF-treated animals were sacrificed at 24, 72, and 240 hr after injection; phenobarbital- and carbon tetrachloride-treated animals were sacrificed 24 hr after the last injection. Control animals, receiving the appropriate vehicle, were also sacrificed at each time point. The doses of BP, phenobarbital, and carbon tetrachloride were so chosen that significant changes in microsomal enzyme activity would be produced by each compound. All animals were sacrificed by exsanguination, and the lungs and livers were excised and homogenized in 0.25 M sucrose with a VirTis 45 homogenizer at 10,000 to 20,000 rpm for 2 min at 0–2\(^\circ\). For whole-organ metal analyses, 4 animals from each test group were sacrificed, and the organs were immediately removed and frozen. Tissue metal analyses were subsequently performed as described below for subcellular fractions. The homogenizer used in this study was equipped with a special tantalum shaft to avoid elemental contamination of the homogenate.

Purified subcellular fractions of both organs were prepared by a differential centrifugation method (9) in an International Model B-35 centrifuge. The fractions were stored at −10\(^\circ\) in metal-free polyethylene vials before elemental analysis. Aliquots of each fraction were analyzed for nitrogen by the automated Kjeldahl method (1). After wet ashing of the subfractions with redistilled nitric acid, the ash residues were treated 3 times with redistilled hydrochloric acid (6 N) and taken to dryness after each treatment. The final hydrochloric acid concentration was approximately 0.3 N. Metal concentrations of the sample solutions were determined by atomic absorption spectrophotometry, with a Perkin-Elmer Model 403 atomic absorption spectrophotometer equipped with a triple-slot burner head, deuterium arc background corrector, and air-acetylene flame. Instrumental conditions as recommended by the manufacturer were generally followed (2). Since the expression of subcellular metal concentrations is rendered difficult by the lack of any biologically meaningful base line, concentrations have been expressed according to the nitrogen content of the subfraction and on estimated fraction volumes to allow comparison with previous work (14).

The 10,000 × g supernatant, containing the microsomal fraction, was used for enzyme activity measurements. BP hydroxylase was determined by the method of Sunderman (12) with an Aminco-Bowman SPF-1000 spectrophotofluorometer. Liver aniline hydroxylase activity was determined by the colorimetric method of Dixon et al. (6), with a Bausch and Lomb Spectronic 20 colorimeter-spectrophotometer.

RESULTS

The subcellular distribution of copper in rat lung after a single 8-mg intratracheal dose of BP is shown in Chart 1. All animals were sacrificed 72 hr after administration of BP. Lung microsomal copper is increased 4-fold over control levels, with no profound changes in the copper concentration of the other subcellular fractions. Since this dose of BP also produces a 6-fold increase in lung BP hydroxylase activity at 72 hr (Table 1), it appears that an increase in the copper content of the microsomal fraction may be associated with increased microsomal enzyme synthesis.

A 4-fold increase in lung microsomal manganese and a 40% depression of nuclear manganese were also produced by this same dose of BP (Chart 2).

Charts 3 and 4 show the effect of BP on the subcellular distribution of nickel and chromium in rat lung. Significant decreases in nuclear, mitochondrial, and microsomal nickel were evident 72 hr after treatment. Chromium is also significantly decreased in these subcellular fractions.

The time course of changes in microsomal copper and nickel, as well as lung BP hydroxylase activity, after a single 8-mg intratracheal dose of BP is summarized in Table 1. BP hydroxylase is expressed as percentage of control activity. Control values have been pooled over all time periods. At 72 hr after treatment, microsomal copper and enzyme activity are increased 4- and 6-fold, respectively, over control levels, and microsomal nickel is maximally depressed. All values are approaching control levels at 240 hr after treatment.

The effect of phenobarbital administration on the subcellular distribution of copper, manganese, and zinc in rat liver is shown in Charts 5, 6, and 7, respectively. A highly significant (p < 0.001) 5-fold increase in liver microsomal copper was found, as well as less significant increases in the copper content of the other cell fractions of treated animals. Manganese and zinc distributions follow this same pattern; i.e., the most dramatic increase in metal concentration is associated with the microsomal fraction. The nickel and chromium levels

\[\text{CONTROL}\]

\[\text{BERYPHREN, 8 mg}\]

\[\text{*Sig. Diff., p < 0.001}\]

\[\text{NUCLEAR}\]

\[\text{MITOCHONDRIA}\]

\[\text{MICROSOMAL}\]

\[\text{SOLUBLE}\]

Chart 1. Copper in rat lung cell fractions 72 hr after intratracheal BP. Each value represents the mean ± S.E. of 8 animals/group.

\(^3\)Mention of commercial products or concerns does not constitute endorsement by the USPHS.
Table 1

Effect of intratracheal BP on microsomal copper, nickel, and BP hydroxylase activity in rat lung

<table>
<thead>
<tr>
<th>Time after BP³ (hr)</th>
<th>No. of animals</th>
<th>Lung microsomal copper (µg/mg)²</th>
<th>Lung microsomal nickel (µg/mg)²</th>
<th>Lung BP hydroxylase activity (% of control)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control⁵</td>
<td>12</td>
<td>0.219 ± 0.046</td>
<td>0.72 ± 0.33</td>
<td>100 ± 12</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>0.264 ± 0.091</td>
<td>&lt;0.01d</td>
<td>188 ± 30d</td>
</tr>
<tr>
<td>72</td>
<td>8</td>
<td>0.555 ± 0.115d</td>
<td>&lt;0.01d</td>
<td>591 ± 42d</td>
</tr>
<tr>
<td>240</td>
<td>8</td>
<td>0.200 ± 0.080</td>
<td>0.25 ± 0.13d</td>
<td>225 ± 15d</td>
</tr>
</tbody>
</table>

* Single intratracheal dose of BP, 8 mg.
* Mean ± S.E. of n animals/group.
* Controls (peanut oil, 0.5 ml/kg) pooled over all time periods.
* Significant difference, p < 0.001, from control values.
* Significant difference, p < 0.01, from control values.

Chart 2. Manganese in rat lung cell fractions 72 hr after intratracheal BP. Each value represents the mean ± S.E. of 8 animals/group.

Chart 4. Chromium in rat lung cell fractions 72 hr after intratracheal BP. Each value represents the mean ± S.E. of 8 animals/group.

Chart 3. Nickel in rat lung cell fractions 72 hr after intratracheal BP. Each value represents the mean ± S.E. of 8 animals/group.

Chart 5. Copper in rat liver cell fractions after phenobarbital. Animals were sacrificed 24 hr after the last dose. Each value represents the mean ± S.E. of 8 animals/group.

of liver subcellular fractions were also examined after phenobarbital; however, no significant difference from control levels was observed with the particular dosage regimen used in this study. Pretreatment with phenobarbital was also associated with a significant elevation of liver microsomal aniline hydroxylase activity (305% of control activity).

For determination of whether the subcellular metal changes produced by pretreatment with phenobarbital and intratracheal BP were characteristic only of the enzyme-responsive tissue, the subcellular metal concentration of a nonresponsive tissue (i.e., no change in enzyme activity) was examined after each inducer. The intratracheal administration of BP produced no significant changes in the subcellular distribution of copper, manganese, zinc, nickel, and chromium in rat liver at any of the time periods studied. Similarly, pretreatment with phenobarbital had no effect on the subcellular distribution of these elements in rat lung.

The effect of the administration of 3 subacute doses of carbon tetrachloride on liver microsomal metals and microsomal aniline hydroxylase activity is summarized in
Microsomal Enzyme Activity and Subcellular Metals

Table 2. A dose of 0.5 ml/kg (p.o.) produced the maximum depression of liver microsomal copper, manganese, and zinc levels from control values. This dose also produced the maximum inhibition of liver aniline hydroxylase activity. Microsomal metal levels and enzyme activity appear to increase at the largest dose studied, 1.0 ml/kg.

Nitrogen was chosen as the base line for expression of subcellular metal concentration, because it is a biologically meaningful parameter. The percentage distribution of whole-organ nitrogen among the various subcellular fractions remained essentially constant, regardless of the treatment used in this study. One exception was the expected increase (20 to 30%) in microsomal nitrogen after phenobarbital, which is a characteristic anabolic effect of this compound on the liver (4). However, none of the metal changes observed in this study could be explained by alterations in the nitrogen content of the subcellular fraction.

In addition, the observed subcellular metal changes did not reflect alterations in whole-organ metal content. Table 3

![Chart 6. Manganese in rat liver cell fractions after phenobarbital. Each value represents the mean ± S.E. of 8 animals/group.](image)

![Chart 7. Zinc in rat liver cell fractions after phenobarbital. Each value represents the mean ± S.E. of 8 animals/group.](image)

Table 2

<table>
<thead>
<tr>
<th>Dose of CCl₄ (ml/kg)</th>
<th>Microsomal metal (µg/mg)</th>
<th>Aniline hydroxylase activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Copper</td>
<td>Manganese</td>
</tr>
<tr>
<td>Control</td>
<td>0.260 ± 0.07</td>
<td>0.185 ± 0.03</td>
</tr>
<tr>
<td>0.25</td>
<td>0.233 ± 0.09</td>
<td>0.307 ± 0.15</td>
</tr>
<tr>
<td>0.50</td>
<td>0.177 ± 0.01e</td>
<td>0.125 ± 0.01d</td>
</tr>
<tr>
<td>1.00</td>
<td>1.200 ± 0.28e</td>
<td>0.560 ± 0.19e</td>
</tr>
</tbody>
</table>

*Carbon tetrachloride, p.o. injection. Animals sacrificed 24 hr after administration.

†Mean ± S.E. of 6 animals/treatment.

‡Significant difference, p < 0.01, from control values.

§Significant difference, p < 0.05, from control values.

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Organ</th>
<th>Copper (µg/mg)</th>
<th>Manganese (µg/mg)</th>
<th>Zinc (µg/mg)</th>
<th>Chromium (µg/mg)</th>
<th>Nickel (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Lung</td>
<td>0.20 ± 0.01</td>
<td>0.03 ± 0.02</td>
<td>1.11 ± 0.19</td>
<td>0.90 ± 0.30</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>BP</td>
<td>Lung</td>
<td>0.19 ± 0.10</td>
<td>0.05 ± 0.04</td>
<td>1.68 ± 0.44</td>
<td>0.75 ± 0.50</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>Control</td>
<td>Liver</td>
<td>0.59 ± 0.17</td>
<td>0.36 ± 0.14</td>
<td>1.88 ± 0.56</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Liver</td>
<td>0.57 ± 0.02</td>
<td>0.31 ± 0.01</td>
<td>1.77 ± 0.19</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>CCl₄, 0.5 ml/kg</td>
<td>Liver</td>
<td>0.61 ± 0.15</td>
<td>0.36 ± 0.20</td>
<td>1.90 ± 0.30</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>CCl₄, 1.0 ml/kg</td>
<td>Liver</td>
<td>0.72 ± 0.20</td>
<td>0.37 ± 0.20</td>
<td>1.91 ± 0.15</td>
<td>0.20 ± 0.09f</td>
<td>0.21 ± 0.10f</td>
</tr>
</tbody>
</table>

*BP, 8 mg, intratracheal injection. Animals sacrificed 72 hr after administration.

†Phenobarbital, 75 mg/kg/day for 3 days. Animals sacrificed 24 hr after last dose.

‡Carbon tetrachloride (CCl₄), p.o. injection. Animals sacrificed 24 hr after administration.

§Mean ± S.E. of 4 animals/treatment.

‖Significant difference, p < 0.05, from control values.

fSignificant difference, p < 0.01, from control values.
summarizes the effects of BP, phenobarbital, and carbon tetrachloride on the whole-organ concentrations of copper, manganese, zinc, nickel, and chromium. Tissue metal levels remained relatively constant after the various pretreatments. Therefore, the subcellular metal changes observed in this study may reflect a redistribution or "intracellular shift" of endogenous metal stores in the target tissue.

DISCUSSION

The results of the present study indicate that the induction of lung BP hydroxylase activity by BP is associated with an increase in the copper and manganese content of the microsomal fraction and a corresponding decrease in the nickel and chromium content of this fraction. These in vivo metal shifts added to the in vitro activating effects of copper and the inhibitory effects of nickel and chromium, described by Dixon et al. (5), suggest that trace metals may play a hitherto undefined role in the regulation of microsomal enzyme activity in rat lung, perhaps by altering the affinity of the substrate for cytochrome P-450 or the specificity of the substrate-hemoprotein complex for the active site on the enzyme molecule (3). The metabolism of BP in rat lung appears to be associated with an increase of activating metals and a decrease of inhibitory metals in the microsomes.

The effect of phenobarbital on the liver microsomal metals, copper, manganese, and zinc, suggests that a similar mechanism may operate in rat liver. The nonspecific increase of liver microsomal metals after phenobarbital may be associated with a generalized increased synthesis of microsomal membranes, which contain significant normal amounts of protein-bound copper and manganese (10).

The effect of a microsomal enzyme inducer on subcellular metal distribution appears to be limited to the enzyme-responsive tissue, since intratracheal BP had no effect on liver subcellular metals, and phenobarbital administration produced no changes in the subcellular metal distribution of rat lung.

The effects of carbon tetrachloride on liver microsomal metals and aniline hydroxylase activity indicate that the inhibition of microsomal enzyme activity is associated with a decrease in certain microsomal metals. This effect appears to be dependent on the dose of carbon tetrachloride and the structural integrity of the liver. Thiers et al. (13) have shown that a toxic dose of carbon tetrachloride (2.5 ml/kg) is associated with subcellular changes in almost all metals examined, especially the alkali metals in the mitochondrial fraction. These latter changes were correlated with a loss of oxidative function of these organelles and were attributed to a loss of structural integrity of hepatic cells. In the present investigation, the highest dose of carbon tetrachloride used (1.0 ml/kg) was also associated with a generalized increase of liver subcellular metals, 24 hr after treatment. This apparent increase in subcellular metal concentration may be explained by the hepatic necrosis and the subsequent increased resistance to hepatic blood flow which have been reported to occur after this dose of carbon tetrachloride (11). This condition would result in a decreased clearance of serum protein bound metals from the damaged liver and an increase in hepatic metal content. Therefore, it is possible that the slight increase in enzyme activity after the highest dose of carbon tetrachloride may be an experimental artifact due to the high levels of activating metals in the microsomal preparation.

Fahim et al. (7) have recently reported that the chronic administration of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) a potent hepatic microsomal enzyme inducer, is associated with a significant increase in total liver copper. Hilderbrand et al. (8) have demonstrated a similar phenomenon after the chronic administration of phenobarbital. In the present study, the acute administration of phenobarbital did not alter whole-organ copper content; however, the microsomal concentration of this element was dramatically increased. It is interesting to speculate that an early event in microsomal enzyme induction may be a redistribution of endogenous activating or inhibitory metals. With prolonged administration of the inducing compound, the lung or liver may require additional activator, which must be mobilized from exogenous stores. This latter hypothetical process may account for the increased whole-liver copper content observed after chronic administration of phenobarbital or 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane.

Our atomic absorption data on the normal subcellular partition of copper, manganese, and zinc in rat liver compare very favorably with previous data reported by Thiers and Vallee (14) in 1957, using emission spectroscopy. In both studies, the pattern of metal distribution in each fraction was characteristic and reproducible. The values reported in this paper for the zinc and manganese content of the soluble fraction are slightly higher than the earlier values reported by Thiers; however, these differences can probably be explained by the increased analytical sensitivity of atomic absorption spectrophotometry for these elements (2). We have been unable to find previous data on the normal subcellular distribution of metals in rat lung for comparison with our results.

The possible role of trace metals in the regulation of microsomal enzyme activity warrants further investigation. Additional work is required to determine whether the subcellular metal changes observed in this study are a consequence of microsomal enzyme induction or inhibition, or whether these changes represent an essential preliminary event in the inductive or inhibitory process.

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