THE BINDING OF BENZO[A]PYRENE TO RAT LIVER PROTEIN AND NUCLEIC ACIDS IN VIVO

by

LARRY STUART GONTOVNICK
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We accept this thesis as conforming to the required standard.

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Department of **Pharmacology**, Faculty of Pharmaceutical Sciences

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date **February 6th**, 1978.
ABSTRACT

In the present study rats were pretreated with agents that are known to affect the activities of the benzo[a]pyrene (BP) metabolizing enzymes in vitro. Also, agents which are known to alter the levels of hepatic glutathione were used. These experiments were carried out in order to determine the effects of enzyme induction, enzyme inhibition, and glutathione levels on the degree of covalent binding of BP to liver macromolecules in vivo. In addition the roles of aryl hydrocarbon hydroxylase (AHH) and epoxide hydratase (EH) can be studied in this way. Since the degree of covalent binding of polycyclic aromatic hydrocarbons (PAH's) and their tumor-initiating ability have been shown to correlate (13), the factors which govern the extent of covalent binding are of major importance.

When $^3$H-BP was administered intraperitoneally to male Wistar rats, a certain amount of the compound was bound irreversibly to liver macromolecules. The degree of irreversible binding was found to be dependent on both the dose of BP administered and the time after its injection. The degree of binding was found to be linearly dependent on the dose of BP between the range of 0.125 and 12.5 μmoles, thereby suggesting that the metabolizing pathways of BP were not saturated at these levels. The study showed the maximum level of binding to occur at 12 to 18 hours after 1.25 μmole of BP, and this fell to 60% of maximum by 48 hours. The BP dosage of 1.25 μmole was employed throughout the study.

The results showed that pretreating rats with SKF 525-A significantly decreased the level of irreversibly bound BP from control levels by about 30%. The decrease in binding after SKF 525-A treatment is in agreement with the evidence that the cytochrome P-450 enzymes are responsible for the activation of BP to reactive intermediates, and that they can be inhibited by this
compound (42). SKF 525-A at 35 mg/kg, a dose which produces inhibition of AHH in vitro (74) did not decrease the binding of BP in vivo. SKF 525-A at 50 mg/kg or higher was required to produce a decrease in binding, indicating the necessity to reach a higher effective hepatic concentration of SKF 525-A to inhibit the irreversible binding of BP in vivo, in contrast to a lesser amount of SKF 525-A required to inhibit BP hydroxylase in vitro.

Oral methadone pretreatment failed to alter the level of BP binding to liver macromolecules. Methadone was found to increase hepatic epoxide hydrolase by 212% in male Wistar rats (66), but in the present study this did not have any influence on the degree of binding of BP in vivo.

3-Methylcholanthrene (3-MC) pretreatment was found to significantly decrease the level of irreversibly bound BP from control by about 30%. The possible causes of this 3-MC induced decrease in binding are discussed. One possibility is that the 3-MC induced decrease could be due to an alteration in the pathways of BP metabolism. The existence in the liver of various forms of cytochrome P-450 (39) along with the evidence that 3-MC induces a spectrally distinct cytochrome P-448 (40,58) could suggest that 3-MC alters BP metabolism to sites on the BP molecule that produce less reactive intermediates, and thereby decreases the degree of binding.

Neither cysteine nor diethyl maleate pretreatment altered the level of irreversibly bound BP from control. The data obtained from these experiments can be explained by one or more of the following mechanisms: no significant depletion of glutathione by BP occurs, there is a lack of a glutathione threshold level for binding to take place, or there is a drastically different role for glutathione than its role in the protection of hepatic macromolecules from alkylation by active metabolites of acetaminophen.

The enzyme-mediated binding of BP to liver macromolecules in vitro and
its inhibition by methadone, SKF 525-A, 3-MC, glutathione, and cysteine was demonstrated and the relevance of these findings towards the present experiments was discussed.

Throughout the study a second population of animals showed binding of BP that was both qualitatively and quantitatively different from the first. The percentage of animals that fell into this 2nd population was 19% (46 out of 250) of all the animals used in the study.

Dr. G.D. Bellward (Supervisor)
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>METABOLIC ACTIVATION OF PAH's TO EPOXIDES</td>
<td>1</td>
</tr>
<tr>
<td>THE PATHWAYS OF BP METABOLISM</td>
<td>6</td>
</tr>
<tr>
<td>FACTORS THAT INFLUENCE THE RATE AND PATHWAYS OF METABOLISM OF THE PAH's</td>
<td>10</td>
</tr>
<tr>
<td>EFFECT OF ENZYME INDUCTION AND INHIBITION ON THE BINDING OF PAH's IN VIVO</td>
<td>12</td>
</tr>
<tr>
<td>THE OBJECTIVES OF THE PRESENT STUDY</td>
<td>12</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>14</td>
</tr>
<tr>
<td>CHEMICALS</td>
<td>14</td>
</tr>
<tr>
<td>ANIMALS</td>
<td>14</td>
</tr>
<tr>
<td>ANIMAL TREATMENTS FOR IN VIVO BINDING EXPERIMENTS</td>
<td>15</td>
</tr>
<tr>
<td>LIVER PREPARATION</td>
<td>15</td>
</tr>
<tr>
<td>DETERMINATION OF IRREVERSIBLE BINDING OF $^3$H-BP IN VIVO</td>
<td>16</td>
</tr>
<tr>
<td>DETERMINATION OF IRREVERSIBLE BINDING OF $^3$H-BP IN VITRO</td>
<td>17</td>
</tr>
<tr>
<td>RESULTS</td>
<td>19</td>
</tr>
<tr>
<td>CONTROL EXPERIMENTS</td>
<td>19</td>
</tr>
<tr>
<td>IRREVERSIBLE BINDING OF $^3$H-BP IN VITRO</td>
<td>19</td>
</tr>
<tr>
<td>EFFECT OF IN VITRO ADDITIONS ON THE IRREVERSIBLE BINDING OF $^3$H-BP</td>
<td>31</td>
</tr>
<tr>
<td>RELATIONSHIP BETWEEN IRREVERSIBLE BINDING IN VIVO AND DOSE OF BP</td>
<td>31</td>
</tr>
<tr>
<td>IRREVERSIBLE BINDING IN VIVO WITH TIME</td>
<td>42</td>
</tr>
<tr>
<td>EFFECT OF PRETREATMENTS ON THE IRREVERSIBLE BINDING OF $^3$H-BP IN VIVO</td>
<td>59</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>78</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>90</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>92</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Conditions for the irreversible binding of (^3)H-benz[a]pyrene to rat liver 10,000 x g supernatant macromolecules <em>in vitro</em></td>
<td>29</td>
</tr>
<tr>
<td>II</td>
<td>The effect of varied levels of the NADPH-generating system cofactors on the irreversible binding of (^3)H-benz[a]pyrene <em>in vitro</em>.</td>
<td>30</td>
</tr>
<tr>
<td>III</td>
<td>The effect of cysteine and diethyl maleate treatments on the degree of irreversible binding of (^3)H-benz[a]pyrene to rat liver macromolecules <em>in vivo</em>.</td>
<td>77</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Possible mechanisms of carcinogenesis by the ultimate electrophilic reactants derived from chemical carcinogens or precarcinogens</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Benzo[a]pyrene metabolism</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Reactions of benzo[a]pyrene by cytochrome P-450 mixed function oxygenases, epoxide hydratase, glutathione transferase, and other conjugating enzymes</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>Control experiments</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>The effect of incubation time on the irreversible binding of $^3$H-benzo[a]pyrene <em>in vitro</em></td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>The effect of protein concentration on the irreversible binding of $^3$H-benzo[a]pyrene <em>in vitro</em></td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>The effect of substrate concentration on the irreversible binding of $^3$H-benzo[a]pyrene <em>in vitro</em></td>
<td>27</td>
</tr>
<tr>
<td>8</td>
<td>The effect of methadone on the irreversible binding of $^3$H-benzo[a]pyrene <em>in vitro</em></td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>The effect of SKF 525-A on the irreversible binding of $^3$H-benzo[a]pyrene <em>in vitro</em></td>
<td>34</td>
</tr>
<tr>
<td>10</td>
<td>The effect of glutathione on the irreversible binding of $^3$H-benzo[a]pyrene <em>in vitro</em></td>
<td>36</td>
</tr>
<tr>
<td>11</td>
<td>The effect of cysteine on the irreversible binding of $^3$H-benzo[a]pyrene <em>in vitro</em></td>
<td>38</td>
</tr>
<tr>
<td>12</td>
<td>The effect of 3-methylcholanthrene on the irreversible binding of $^3$H-benzo[a]pyrene <em>in vitro</em></td>
<td>40</td>
</tr>
<tr>
<td>13</td>
<td>The effect of dose on the irreversible binding of $^3$H-benzo[a]pyrene to total liver protein and nucleic acids <em>in vivo</em> expressed as pmoles bound/mg protein</td>
<td>43</td>
</tr>
<tr>
<td>14</td>
<td>The effect of dose on the irreversible binding of $^3$H-benzo[a]pyrene to rat liver macromolecules <em>in vivo</em> expressed as pmoles bound/gram wet weight of liver</td>
<td>45</td>
</tr>
<tr>
<td>15</td>
<td>The dose-irreversible binding curve of $^3$H-benzo[a]pyrene in the second population of rats expressed as pmoles bound/mg protein</td>
<td>47</td>
</tr>
<tr>
<td>16</td>
<td>The dose-irreversible binding curve of $^3$H-benzo[a]pyrene in the second population of rats expressed as pmoles bound/gram wet weight of liver</td>
<td>49</td>
</tr>
<tr>
<td>17</td>
<td>The effect of methadone treatment on the irreversible binding of $^3$H-benzo[a]pyrene <em>in vivo</em> expressed as pmoles bound/gram wet weight of liver</td>
<td>51</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>18</td>
<td>The effect of methadone treatment on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo} expressed as pmoles bound/mg protein.</td>
<td>53</td>
</tr>
<tr>
<td>19</td>
<td>The effect of methadone treatments on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo} in the second population of rats expressed as pmoles bound/gram wet weight of liver.</td>
<td>55</td>
</tr>
<tr>
<td>20</td>
<td>The effect of methadone treatment on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo} in the second population of rats expressed as pmoles bound/gram wet weight of liver.</td>
<td>57</td>
</tr>
<tr>
<td>21</td>
<td>The effect of corn oil and 3-methylcholanthrene treatments on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo} expressed as pmoles bound/gram wet weight of liver.</td>
<td>60</td>
</tr>
<tr>
<td>22</td>
<td>The effect of corn oil and 3-methylcholanthrene treatments on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo} expressed as pmoles bound/mg protein.</td>
<td>62</td>
</tr>
<tr>
<td>23</td>
<td>The effect of corn oil and 3-methylcholanthrene treatments on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo} in the second population of rats expressed as pmoles bound/gram wet weight of liver.</td>
<td>66</td>
</tr>
<tr>
<td>24</td>
<td>The effect of corn oil and 3-methylcholanthrene treatments on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo} in the second population of rats expressed as pmoles bound/mg protein.</td>
<td>68</td>
</tr>
<tr>
<td>25</td>
<td>A comparison of the effects of corn oil and 3-methylcholanthrene (3-MC) treatments on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo} in animals injected with the compound at either 24 or 48 hours after the last dose of either corn oil or 3-MC.</td>
<td>70</td>
</tr>
<tr>
<td>26</td>
<td>A comparison of the effects of corn oil and 3-methylcholanthrene (3-MC) treatments on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo} in the second population of rats injected with the compound at either 24 or 48 hours after the last dose of either corn oil or 3-MC.</td>
<td>72</td>
</tr>
<tr>
<td>27</td>
<td>The effect of SKF 525-A treatment on the irreversible binding of $^3$H-benzo[a]pyrene \textit{in vivo}.</td>
<td>74</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AHH  aryl hydrocarbon hydroxylase
BP   benzo[a]pyrene
dpm  disintegrations per minute
EH   epoxide hydratase
GSHT glutathione-S-transferase
i.p. intraperitoneally
i.v. intravenously
3-MC 3-methylcholanthrene
NADP⁺ niotinamide adenine dinucleotide phosphate
NADPH nicotinamide adenine dinucleotide phosphate (reduced)
PAH  polycyclic aromatic hydrocarbon
S.E.M. the standard error of the mean
TCA  trichloroacetic acid
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INTRODUCTION

Chemical carcinogenesis was first documented by the British surgeon Sir Percival Pott, in 1775, who called attention to the high incidence of scrotal cancer in the chimney sweeps of London and attributed this to their constant contact with coal tar and soot (1). Following this observation by about 150 years was the isolation of a polycyclic aromatic hydrocarbon (PAH), benzo[a]pyrene (BP), from coal tar and the demonstration that it produced cancer on the skin of mice (2).

![Benzo[a]pyrene structure]

BP, the most commonly found PAH, is a powerful carcinogen which is present in tobacco smoke (3), is a contaminant of the urban environment (4), and is suspected of contributing to the increasing incidence of cancer of the respiratory tract in man (5).

METABOLIC ACTIVATION OF PAH'S TO EPOXIDES

Many classes of chemical carcinogens become covalently bound to DNA, RNA and proteins of the cells in target tissues (6). It has been known since 1951 that carcinogenic PAH's are covalently bound to mouse skin proteins (7), mouse skin DNA (8), and to DNA, RNA, and proteins of transformable rodent cells in culture (9).

In 1969, it was discovered that PAH's, which did not bind covalently to DNA in the test tube, were bound covalently to DNA after incubation of the PAH
in the presence of endoplasmic reticulum (microsomes) and NADPH (10). These experiments showed the requirement for enzymatic activation in order for PAH's to become covalently bound to DNA. More recently, activation of BP by the nuclear envelope has also been reported (11).

The covalent interaction described is thought to be critical to the carcinogenic process. The activated or ultimate carcinogens are usually electrophilic intermediates that react with nucleophilic sites of vital macromolecules in cells, thereby initiating the process of carcinogenesis. This has been interpreted generally to mean that the crucial, causative, chemical event is a two-electron reaction resulting in a stable, covalent bond between the carcinogen and the suspected target molecule, DNA (12). A preliminary study indicated a correlation between the tumor-initiating ability of several hydrocarbons and their ability to bind covalently to DNA (13). Thus the degree of binding of a carcinogen is of ultimate importance. Figure 1 shows the basic hypothesis.

Figure 1. Possible mechanisms of carcinogenesis by the ultimate carcinogenic electrophilic reactants derived from chemical carcinogens or precarcinogens (14).
Boyland, in 1950, suggested that epoxides were formed as intermediates in the metabolism of PAH's and that these intermediates were involved in the carcinogenic activity shown by many hydrocarbons (15). Later work indicated (a) that epoxides were formed as metabolites of hydrocarbons in model systems in vitro (16), (b) that these compounds could bind covalently with cellular proteins and nucleic acids (17), and (c) that many of them were biologically active and could induce mutations in mammalian cells (18), bacteria (19), and bacteriophage (20), malignant transformation in rodent cells in culture (21), and cancer in experimental animals (22).

The incubation of BP with the microsomal fraction, oxygen, and NADPH produced the K-region epoxide, benzo[a]pyrene 4,5-oxide (23). The K-region epoxide of BP reacted covalently with nucleic acids in vitro (24), was shown to be mutagenic in bacterial (25) and mammalian (18) cells, and could transform various cells in culture (26). K-region epoxides were tested in cell culture and found to be more effective in causing neoplastic transformation than were the parent hydrocarbons (27). However, attempts to demonstrate their increased carcinogenic potential in vivo were unsuccessful and BP-4,5-oxide was found to be a weak carcinogen in comparison to BP (28).

Benzo[a]pyrene 4,5-oxide
There is increasing evidence that the K-region epoxide of BP is not the major reactive intermediate in vivo (29). Studies have suggested that the major reactive intermediates in vivo are the \(7,8\)-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene diol epoxide metabolites (30). Two isomers of this compound have been synthesized (31).

\[
\text{(±) -7,8α-dihydroxy-9\beta,10α-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene (Diol Epoxide 1)}
\]

\[
\text{(±) -7β,8α-dihydroxy-9α,10α,epoxy-7,8,9,10-tetrahydro-benzo a pyrene (Diol Epoxide 2)}
\]

It has been shown that the diol epoxides of BP react with DNA to give a product which is chromatographically indistinguishable from that obtained by incubating hamster embryo cells with BP (32). It has also been shown that the fluorescence spectrum of the BP diol-epoxide conjugate with DNA resembles that of the DNA conjugates from BP-treated mouse skin (33) and from interaction with BP-7,8-dihydrindiol mediated by rat liver microsomes (34). Grover and Sims showed that the hydrocarbon-deoxyribonucleoside products formed in BP-treated human bronchial mucosa and mouse skin were indistinguishable from those that were formed when 7,8-dihydro-7,8-dihydroxy-BP-9,10-oxide reacted with DNA in solution. The same hydrocarbon-DNA products were also found in hydrolysates of DNA from mouse skin treated with 7,8-dihydro-7,8-dihydroxy-BP (35). Levin studied mouse skin tumour development with BP and the 4,5-, 7,8-, and 9,10- BP
epoxides. He found that of these epoxides studied, only BP-7,8-oxide was highly carcinogenic, although less carcinogenic than BP (36). Additional studies revealed that BP-7,8-dihydrodiol was considerably more carcinogenic than BP-7,8-oxide (37). Therefore, the two metabolic precursors of the diol epoxides are potent carcinogens. Both diol epoxide 1 and diol epoxide 2 were found to be highly mutagenic toward bacterial and mammalian cells (38).

Weibel, using different forms of cytochrome P-450 from solubilized rabbit liver microsomes showed oxygenation of BP at different positions depending on the form of cytochrome P-450 used. He also showed that some forms produced diols and others did not (39). Therefore, the specificities of different forms of cytochrome P-450 may channel BP metabolism into the various activation and detoxification pathways and thereby help determine the carcinogenic activity of these compounds. It should also be noted that the PAH's induce the formation of a cytochrome spectrally different from cytochrome P-450, namely cytochrome P-448 (40). Also, Oesch has shown that the addition of homogeneous epoxide hydratase (EH) has a different effect on BP mutagenicity if BP is activated by microsomal preparations from 3-methylcholanthrene (3-MC)-pretreated mice (41). Thus, it appears that the relative formation of one or more epoxides in vivo will depend on the types and quantities of the various cytochromes present at the site of metabolism. Species and tissue differences in the pathways of BP metabolism and levels of metabolites produced could also be explained by the different forms of cytochrome P-450 present.
THE PATHWAYS OF BP METABOLISM

It is now established that the initial step in the metabolism of PAH's is carried out by the "mixed function oxygenases", enzymes that are NADPH-dependent and catalyze the incorporation of molecular oxygen into the substrate molecules (42). The oxygenases are involved in the metabolism of most foreign compounds including drugs and insecticides as well as in the metabolism of many endogenous substances such as steroids (43).

Figure 2 shows the known routes of BP metabolism (44). The sequence of enzyme action can be outlined as follows. The initial oxygenation is catalyzed by the mixed function oxygenases, which contain multiple forms of cytochrome P-450. This results in the formation of epoxides at the 4,5; 7,8; and 9,10 positions. The most stable of these, the 4,5-oxide, has been isolated under in vitro conditions and the 7,8- and 9,10-oxides are likely intermediates (45). Their formation can be deduced from the presence of all three of the corresponding dihydrodiols. These are formed by the action of epoxide hydrolase on the oxide intermediates (46). Evidence of this mechanism is the finding that an epoxide hydrase inhibitor, 1,1,1-trichloropropylene oxide, eliminates dihydrodiol formation (46), and the readdition of a partially purified epoxide hydratase results in the appearance of the dihydrodiols (47). Phenols and quinones can either be formed non-enzymatically from the oxide intermediate or be the result of a direct oxygenation independent of epoxide intermediacy.

The mixed function oxygenase, aryl hydrocarbon hydroxylase (AHH), is the primary catalytic attack on the PAH's. Enzyme purification studies have shown that this system is composed of multiple forms of the enzyme (39). The various forms of cytochrome P-450 have different catalytic activity with respect to the formation of the various BP metabolites. The experimental
Figure 2. Benzo[a]pyrene metabolism. Drawn Structures are metabolites that have been isolated and characterized, with the exception of the 9,10- and 7,8-epoxides, whose presence is confirmed by enzymatic diol products and nonenzymatic phenol formation. Bracketed figures are possible or probable metabolites not yet confirmed. (44)
evidence suggests that each form of the cytochrome P-450 may have separate site preferences on the BP molecule, which results in oxygenation at different positions. Thus the specific form of the cytochrome may relate to the detoxification or activation pathways of metabolism.

A second major enzyme system involved in PAH metabolism is epoxide hydratase. Hydrocarbon epoxides are metabolized by the epoxide hydratase present in cells of many tissues in the body. The enzymes appear to be located exclusively on the smooth endoplasmic reticulum of cells and are therefore found in the microsomal fractions when cellular components are separated by centrifugation. The epoxide hydrase is located in close proximity to only some forms of cytochrome P-450 (39, 48). Unlike the microsomal oxygenase, the epoxide hydratases do not require NADPH or, as far as is known, any other cofactor (48). This enzyme has been assayed using both styrene oxide and BP-4,5-oxide as substrates. Kapitulnik, using human liver epoxide hydratase showed that the rates of hydration of the 4,5-, 7,8-, and 9,10-oxides were similar and that the strong correlation \( r > 0.95 \) between the hydration of these and other epoxides and the hydration of styrene oxide indicated the presence of a single epoxide hydratase (49). He also suggested that a single epoxide could be predictive of the capacity to hydrate epoxides from a large number of foreign chemicals.

Another enzyme system involved in BP metabolism is the glutathione-S-transferase (GSHT), which converts the epoxide intermediates to water-soluble conjugates (50). This enzyme is present in the soluble fraction of rat liver preparations and catalyzes the reactions of glutathione, an endogenous tripeptide, with certain organic substrates. Non-enzymatic conjugation of glutathione has also been demonstrated (51). It has been shown in vitro that neither cysteine, N-acetylcysteine nor oxidized glutathione could be substituted for reduced glutathione to produce conjugates of 3,4-dichloronitro-benzene (52).
glutathione conjugate of the diol-epoxide of benzanthracene has been isolated (53).

In addition, there are reports of the presence of different BP conjugates in urine and bile. It has been suggested that these may be glucuronides and/or sulfates (54). BP-3-yl hydrogen sulphate (the sulphate ester of 3-hydroxy-BP) has been identified from metabolites produced by both rat liver 105,000 x g supernatant and human, hamster and rat lung cultures (55). It has also been reported that a series of hydroxylated metabolites of BP including phenols, dihydrodiols and epoxides form glucuronide conjugates in the presence of UDP-glucuronic acid and microsomes (56). The conjugation of BP-4,5-oxide, however, may be via the dihydrodiol which is formed by BP-4,5-oxide hydratase, another microsomal enzyme (56).

Figure 3 shows the reactions of BP by cytochrome P-450 mixed function oxygenases, epoxide hydratase, GSHT, and other conjugating enzymes (57).

Figure 3. Reactions of benzo[a]pyrene by cytochrome P-450 mixed function oxygenases, epoxide hydratase, GSH transferase, and other conjugating enzymes (57).
FACTORS THAT INFLUENCE THE RATE AND PATHWAYS OF METABOLISM OF THE PAH'S

When animals are treated with any one of a large number of organic compounds, the levels of the microsomal oxygenases in the liver and in many other organs, such as lung, kidney, skin and small intestine are raised (58). The effects of pretreating animals with enzyme inducers on the in vitro hepatic metabolism of PAH's was first described by Conney et al. (59), who treated rats with PAH's and showed increases in the level of hepatic AHH (BP hydroxylase). Compounds other than PAH's will also induce the oxygenase. They include compounds with such diverse structures as phenobarbital, aminopyrene, chlordane, and rifampin (60, 61, 62). The compound widely used as an inhibitor of AHH is SKF 525-A (63). This compound when administered intraperitoneally into rats produces a marked decrease in hepatic AHH activity. Benzoflavone is a widely employed compound that will inhibit AHH in vitro (64).

Epoxide hydrase is also inducible by pretreatment with phenobarbital and PAH's (48). Induction, however, is small as compared with the corresponding increases in the levels of AHH. A large number of compounds have been described that will inhibit the hydration of styrene oxide in vitro. Examples of inhibitors of this enzyme are cyclohexane oxide and 3,4-dihydronaphthalene (65). Bellward et al. showed that in male Wistar rats treated with methadone there was an increase in hepatic epoxide hydratase activity without an increase in the hepatic AHH activity (66). This marked the first case of an agent specifically increasing epoxide hydratase activity without affecting AHH.

PAH's and phenobarbital treatment are known to induce the GSHT activity in the rat liver (51).

The role of glutathione has been studied extensively by Mitchell and co-workers and their data indicate that in animals glutathione is essential for the protection of thiol and other nucleophilic groups in proteins and
nucleic acids from a variety of toxic drug metabolites and other alkylating agents (67). These workers have postulated that the hepatotoxicity of agents such as acetominaphen, isoniazid, bromobenzene and furosemide could be due to metabolic activation of these compounds to reactive intermediates which bind covalently to hepatic macromolecules, thus leading to cell necrosis (68). The effects of enzyme induction, enzyme inhibition, and the role of glutathione on the covalent binding of these agents to liver macromolecules in vivo has been studied thoroughly (68).

The importance of enzyme induction, enzyme inhibition and glutathione on the metabolism and binding of PAH's in vitro have been well documented. Recent studies on the metabolism of BP by hepatic microsomal fractions from normal and 3-MC treated rats have shown that the amounts of the non-K-region metabolites, the 7,8- and the 9,10- dihydrodiols were increased to greater extents than are the phenols and the K-region dihydrodiol when livers from 3-MC-treated animals were used (69). Liver microsomes from 3-MC pretreated rats markedly increased the binding of BP to cellular macromolecules (70). It has also been shown that epoxide hydratase inhibitors present in the incubation tube will also increase the binding of BP to cellular macromolecules (46). King showed that glutathione added to the incubation tube markedly decreased the binding of BP to added DNA (70). Therefore, the enzymatic activities of AHH, epoxide hydratase and GSHT, along with the glutathione levels in the cell could play important roles in the metabolism and binding of BP to cellular macromolecules in vivo.

The factors which will determine the degree of binding of a given epoxide to cellular macromolecules in vivo will be (a) its rate of formation, (b) its rate of enzymatic conversion into the related dihydrodiol, (c) its rate of enzymatic and non-enzymatic conversion into the related glutathione conjugate,
and (d) the rate at which it will alkylate cellular macromolecules. Another important factor, mentioned before, is the relative abundances of the various types of cytochrome P-450, which will govern which site on the BP molecule will undergo oxidation.

**EFFECT OF ENZYME INDUCTION AND INHIBITION ON THE BINDING OF PAH's IN VIVO.**

Since the binding of PAH's is thought to be the prime event in the carcinogenic process, the factors that affect the levels of binding of PAH's in vivo are of extreme importance. Unfortunately, little work in this area has been carried out. The metabolism and covalent binding of BP has been determined in isolated perfused lungs of sham- and cigarette smoke-exposed and 3-MC pretreated male Wistar rats (71). After intratracheal instillation of $^3$H-BP, the amount of covalently bound radioactivity was significantly increased in the lungs of animals exposed to cigarette smoke and in the lungs of 3-MC treated animals compared to the corresponding sham-exposed animals. The total amount of dihydrodiols formed in the lung of smoke-exposed rats was 5 times greater than in the sham-treated animals, with the 4,5-, 7,8-, and 9,10-dihydrodiols each showing 5-fold increases. The amount of 3-hydroxy-BP was also increased.

**THE OBJECTIVES OF THE PRESENT STUDY**

In the present study we are utilizing the male Wistar rat liver as our model system to study the effect of enzyme induction, enzyme inhibition and glutathione levels on the binding of BP to hepatic macromolecules in vivo, after its i.p. administration. The liver is employed in this study for the following reasons. Firstly, the enzymatic effects in vitro of the inducers and inhibitors of the BP metabolizing enzymes that we are using have been studied most extensively in the liver. Secondly, the opportunity to study the effect of selective induction of epoxide hydratase by methadone is limited
to its site of induction, the liver; and thirdly, hepatic glutathione function and agents which affect its function have been well documented in this tissue (72, 73).

The carcinogenic activity of BP is thought to be due to the covalent reaction of metabolically formed epoxides with target macromolecules such as DNA, RNA, and protein (12). We are utilizing agents, which have been shown in vitro to affect the pathways involved in the fate of these epoxides, as tools to study the factors that influence the in vivo binding of BP. SKF 525-A has been shown to decrease BP hydroxylase, the mono-oxygenase responsible for the formation of epoxide intermediates (74). Methadone pretreatment showed a selective increase in epoxide hydratase activity, the enzyme responsible for the further transformation of epoxides to dihydrodiols (66). 3-MC increased the activity of BP-hydroxylase as well as induced the formation of a spectrally different cytochrome, namely cytochrome P-448 (40, 58). It also increased EH and GSHT activities, but to a lower degree than the increase in AHH activity (48, 51). Cysteine is a precursor in the biosynthesis of glutathione (73) and diethyl maleate depletes hepatic glutathione levels (72). Therefore, by using these agents we can study the roles of AHH, EH and glutathione on the binding of BP in vivo.
MATERIALS AND METHODS

CHEMICALS:

$^3$H-BP (generally labelled; specific activity 8.3 and 40 Ci/m mole) was obtained from Amersham corporation, (Toronto, Ont.). Methadone·HCl was obtained through the Canadian Department of Health and Welfare. SKF 525-A ((2-diethyl-amino ethyl-2,2-diphenylvalerate·HCl) was a gift from Smith, Kline and French Labs., (Montreal, Que.). 3-MC, diethyl maleate and BP were supplied by Eastman Organic Chemicals, (Rochester, N.Y.); MgCl$_2$·6H$_2$O by British Drug House, (Toronto, Ont.); NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glutathione (reduced) and bovine serum albumin by Sigma Chemical Co., (St. Louis, Mo.); L-(+) -cysteine hydrochloride by Fisher Scientific Co., (Fair Lawn, N.J.). All other reagents and solvents were the best available commercial grades.

The purity of the $^3$H-BP was 98% as shown by thin-layer chromatography on silica gel, (Eastman, Rochester, N.Y.) using two solvent systems (benzene-hexane, 1:15 and benzene-ethanol, 9:1). The non-labelled BP was dissolved in benzene, filtered and recrystallized from cold methanol.

ANIMALS:

Wistar rats, males (200-300gm), obtained locally (University of British Columbia) were employed throughout the study. For each experiment, control and treated rats were matched for age and weight with minimal variation. The animals, 4 per cage, were allowed to equilibrate for at least one week under controlled conditions (22°C, lights on 0600 to 2000 hours). 'Lobund' grade corn-cob bedding, (Paxton Processing Ltd., Paxton, I11.) was used. Purina Lab Chow and tap water were supplied ad libitum, unless otherwise stated.
ANIMAL TREATMENTS FOR IN VIVO BINDING EXPERIMENTS

\( ^3\text{H-BP} \) diluted with non-labelled BP was administered intraperitoneally (1.25 \( \mu \text{mole} \) \( ^3\text{H-BP}, 125 \mu\text{Ci} \) in 0.5 ml, corn oil). The dose of \( ^3\text{H-BP} \) was altered by varying the amount of non-labelled BP, the volume and quantity of radioactivity injected remaining constant. Animals were sacrificed at the indicated times after \( ^3\text{H-BP} \).

\( 3\text{-MC} \) in corn oil was injected i.p. at 20 mg/kg for 2 consecutive days. Control animals received corn oil only. Animals were administered \( ^3\text{H-BP} \) 24 hours after the last \( 3\text{-MC} \) injection, unless otherwise stated.

\( \text{SKF 525-A} \) (single dose at 35 mg/kg, 50 mg/kg or 75 mg/kg) was given i.p. in saline. Control animals received saline only. \( ^3\text{H-BP} \) was administered 3 hours after \( \text{SKF 525-A} \).

Diethyl maleate in corn oil (0.6 ml/kg i.p.) was injected 30 minutes before administration of \( ^3\text{H-BP} \). Control animals received corn oil only.

\( \text{L-}(+)\text{-cysteine-HCl} \) in saline (150 mg/kg i.p.) was administered 5 minutes before \( ^3\text{H-BP} \). Control animals received saline alone.

The pretreatment solutions were administered at a volume to animal weight ratio of approximately 2.3 ml/kg.

Methadone·HCl was dissolved in the drinking water. The dosage of methadone was increased once a week, until 4 weeks; as they developed a tolerance to the drug. All methadone doses given as mg/kg are for a 24 hour period. Animals were maintained on methadone up until the time of sacrifice in order to prevent the development of abstinence symptoms.

LIVER PREPARATION

All animals were stunned by a blow on the head, killed by decapitation and bled. The livers were then perfused with ice-cold 1.15% KCl in situ and
DETERMINATION OF IRREVERSIBLE BINDING OF $^3$H-BP IN VIVO

Quantitation of the irreversible binding of $^3$H-BP to total liver protein and nucleic acids, after its i.p. injection into rats, was determined according to the method of Jollow et al. (75). Five grams of liver tissue were minced and homogenized in 20 ml of ice-cold 1.15% KCl, using a motor-driven Potter-Elvehjem homogenizer. Duplicate 1 ml aliquots of each homogenate, equivalent to 0.25 gm of liver, were then added to 2 ml of 0.9 M trichloroacetic acid (TCA), in disposable culture tubes, to precipitate the protein and nucleic acids. The tubes were then vortexed and centrifuged (International centrifuge, size 2, model EXD) at room temperature, at 1,000 x g, for 10 min. The supernatant was discarded and the protein resuspended in 3 ml of 0.6 M TCA, using a vortex shaker and stainless steel spatula to improve mixing. The tubes were centrifuged again at 1,000 x g for 3 min and the supernatant was discarded. The protein was resuspended in 0.6 M TCA twice more and after the supernatant was discarded on the last 0.6 M TCA wash, the protein was resuspended in 3 ml of 80% methanol, mixed with a spatula, as above, centrifuged at 1,000 x g for 3 min and the supernatant discarded. The procedure was repeated until no further radioactivity could be removed. Further washings with acetone, chloroform and ether failed to remove additional radioactivity. The extracted protein was then dissolved in 5 ml of 1N NaOH and a 0.5 ml aliquot, equivalent to 0.025 gm of liver, was taken from each sample and added along with 0.5 ml of 1.2 N HCl to 15 ml of Biofluor scintillation fluid, (New England Nuclear, Dorval, Que.) and counted in a Searle Mark III liquid scintillation counter. The 1.2 N HCl was needed to decrease chemiilluminescence in the samples (76). Radioactivity was corrected for background and for quenching. Quench standards
were prepared using $^3$H-toluene, (New England Nuclear, Dorval Que.), Biofluor and acetone as the quenching agent. The vials were sealed with wax. The counting efficiency for tritium in the liquid scintillation counter ranged from 46% to 48%. Protein concentration was determined by the method of Sutherland et al. (77) as modified by Robson et al. (78), using crystalline bovine serum albumin as the protein standard.

**DETERMINATION OF IRREVERSIBLE BINDING OF $^3$H-BP IN VITRO**

For quantitation of the irreversible binding of $^3$H-BP in vitro, 5 grams of liver tissue were minced and homogenized in 20 mls. of ice-cold 1.15% KC1, for 1 min. 15 sec., using a motor-driven glass-teflon homogenizer. The homogenate was then centrifuged, (International refrigerated centrifuge, Type B-20) at 4°C, at 10,000 x g, for 10 mins. and the supernatant diluted 1:5 with the KCl solution, and this was used as the enzyme suspension, unless indicated otherwise.

The incubation was done in a total of 5 ml. containing the following: 1 ml. of enzyme preparation, equivalent to 0.05 gm of liver, the NADPH-generating system in 3.9 mls of 0.2 M phosphate buffer, pH 7.4 and 25 μmoles of glucose-6-phosphate, 12.5 μmoles of NADP +, 6.25 μmoles of MgCl 2 and 2.0 units of glucose-6-phosphate dehydrogenase) and 1.015 μmoles of $^3$H-BP (2.221 x 10 6 dpm) in 50 μl of acetone.

Drug additions were made prior to the incubation. SKF 525-A, methadone·HCl, cysteine·HCl or glutathione (reduced) was added in 50 μl of 0.2 M phosphate buffer. Control tubes received 50 μl of the buffer. 3-MC was added in 50 μl of amyl alcohol and the control tubes for the 3-MC experiments also received 50 μl of amyl alcohol.

The blanks contained 1 ml of enzyme suspension boiled for 10 min. at 100°C. Blanks which contained boiled enzyme and the highest concentration of
The drug studied were also used.

The tubes were preincubated for 1 min, the enzyme suspension added at 0 time and the tubes incubated for 50 min. Both the preincubation and incubation were carried out at 37°C in a shaking water bath. The reactions were stopped at the desired time by adding 1 ml of 0.9 M TCA. The precipitated protein and nucleic acids were vortexed and centrifuged for 10 min at room temperature at 1,000 x g. The supernatant was discarded and the precipitate extracted twice with 0.6 M TCA, 3 times with 80% methanol, 3 times with acetone and at least 3 times with ether. Extractions were carried out by adding 3 ml of the appropriate solvent to the precipitate, vortexing, centrifuging at room temperature at 1,000 x g for 3 min and pouring off the supernatant. It was found that by further extractions no additional radioactivity could be removed from the precipitate.

The precipitate was then dissolved in 5 ml of 1N NaOH. A 0.5 ml aliquot, equivalent to 0.005 gm liver, and 0.5 ml of 1.2 N HCl were added to 15 ml of Biofluor and counted as previously described.

All calculations were performed on a Wang 600 calculator. The student's t-test for unpaired sample means was used for all comparisons. The level of significance chosen was at p<0.05. The linear regression analysis was performed and the correlation coefficient for the regression line calculated when a linear relationship was statistically tested.
RESULTS

CONTROL EXPERIMENTS

Figure 4 shows the degree of irreversible binding in three different control experiments. In the first experiment animals were sacrificed immediately after $^3$H-BP administration. The level of binding was found to be about 3 pmoles/gram wet weight of liver. In the second experiment $^3$H-BP (192.5 pmoles) or 770 pmoles/gram wet weight of liver; 40,000 dpm) in 100 $\mu$l of corn oil was added to the TCA precipitated aliquot of a typical liver homogenate. This quantity of $^3$H-BP corresponded to the average amount of radioactivity found in a 1 ml aliquot of liver homogenates of 4 corn oil pretreated rats injected with $^3$H-BP and sacrificed 13 hours later. The amount of $^3$H-BP irreversibly bound to total liver protein and nucleic acids after the extraction methods was found to be 16 pmoles/gram wet weight of liver. In the third experiment $^3$H-BP in 200 $\mu$l corn oil was added to a typical liver homogenate and 1 ml aliquots containing 192.5 pmoles of $^3$H-BP (40,000 dpm) or 770 pmoles/gram wet weight of liver were precipitated in TCA. After the extraction procedure the level of irreversibly bound $^3$H-BP was found to be 8 pmoles/gram wet weight of liver. The irreversible binding in those corn oil treated animals was 276 pmoles/gram wet weight of liver.

IRREVERSIBLE BINDING OF $^3$H-BP IN VITRO

$^3$H-BP (1.015 $\mu$mole, 2.221 x $10^6$ dpm) was incubated at 37°C for varying lengths of time in the presence of an NADPH-generating system and the 10,000 $\times$ g liver supernatant. Figure 5 indicates that linearity of binding to rat liver protein and nucleic acids was a function of incubation time. The correlation coefficient of the regression line was 0.974. Dependence of the binding on protein concentration is shown in figure 6. The binding was linear up to the
Figure 4. Non-specific irreversible binding. A: four rats were sacrificed immediately after $^3$H-benzo [a]pyrene ($^3$H-BP) injection (1.25 μmole; 125 μCi) in 0.5 ml corn oil. B: four 1 ml aliquots from each of two liver homogenates were precipitated in trichloroacetic acid (TCA) and $^3$H-BP added (192.5 μmole; 40,000 dpm) in 100 μl corn oil. C: $^3$H-BP was added to two separate liver homogenates; four 1 ml aliquots containing 192.5 μmole $^3$H-BP (40,000 dpm) were taken from each homogenate and precipitated in TCA. Values represent irreversible binding in the extracted pellets.
$^{3}\text{H-BP BOUND}$

pmoles/gram wet weight of liver $\pm$ s.e.m. ($n$)
The effect of incubation time on the irreversible binding of \(^3\)H-benzo[a]pyrene (\(^3\)H-BP) in vitro. Reaction tubes containing \(^3\)H-BP (1.015 \(\mu\)mole; 2.221 \(\times\) \(10^5\) dpm), the NADPH-generating system and the 10,000 x g supernatant equivalent to 0.05 gram of liver were incubated at 37°C for varying periods of time. Blanks contained boiled enzyme. Values are corrected for non-specific binding and are the averages of duplicates. The correlation coefficient of the regression line was 0.974.
$^{3}H$-BP bound (nmol/gram wet weight of liver) vs. Incubation time (minutes)
Figure 6. The effect of protein concentration on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vitro. Reaction tubes containing $^3$H-BP (1.015 µmole; $2.221 \times 10^6$ dpm), the NADPH-generating system and the 10,000 x g. supernatant were incubated at 37°C for 50 mins. Blanks contained boiled enzyme. Values are corrected for non-specific binding and are the averages of duplicates.
10,000 x g supernatant protein content equivalent to 0.05 gram wet weight of liver. The correlation coefficient of a regression line from the origin to the abscissa value of 0.05 had a correlation coefficient of 0.989. Figure 7 shows the effect of substrate concentration on the binding reaction. Subsequently, all reactions were carried out for 50 minutes, containing 1.015 μmole 3H-BP (2.221 x 10^6 dpm) and a diluted 10,000 x g supernatant equivalent to 0.05 gram wet weight of liver.

To determine the enzymatic nature of the binding reaction, experiments were carried out under N₂ atmosphere and without the NADPH-generating system. These results are shown in Table I. The presence of a N₂ atmosphere decreased the binding by 75% of its control value. Deleting the NADPH-generating system reduced the binding to 40% of its control value. Table II shows the effects of increasing some or all of the cofactors of NADPH-generating system. Increasing the amount of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP⁺ or all of these cofactors at once showed no statistically significant increase of BP binding from control.

After the extraction procedures, radioactivity was present in the boiled enzyme blanks. A higher level of radioactivity was observed if active enzyme, without the NADPH-generating system added, was used as blanks. Boiled protein blanks done under N₂ or without the NADPH-generating system showed the same level of radioactivity as the boiled enzyme blanks done under normal conditions. The irreversible binding of 3H-BP to 10,000 x g supernatant protein and nucleic acids refers only to enzyme-mediated binding. All values have been corrected for non-specific or physical binding. The physical binding was shown to increase with increasing amounts of either 3H-BP or protein.
Figure 7. The effect of substrate concentration on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vitro. Reaction tubes containing $^3$H-BP at various concentrations, the NADPH - generating system and the 10,000 x g supernatant equivalent to 0.05 gram of liver were incubated at 37°C for 50 mins. Blanks contained boiled enzyme. Values are corrected for non-specific binding and are the averages of duplicates.
$^{3}\text{H-BP BOUND}$
nmoles/gram wet weight of liver/min

$^{3}\text{H}$-benzo[a]pyrene (μmoles)

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
**TABLE 1.**

Conditions for the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) to rat liver 10,000 x g supernatant macromolecules in vitro.

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>DPM$^a$</th>
<th>DPM MINUS BLANK</th>
<th>IRREVERSIBLY BOUND $^3$H-BP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>nmol/g wet weight/minute</td>
</tr>
<tr>
<td>blank</td>
<td>617</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>control</td>
<td>1273</td>
<td>656</td>
<td>1.199</td>
</tr>
<tr>
<td>N$\textsubscript{2}$ atmosphere</td>
<td>781</td>
<td>164</td>
<td>0.299$^f$</td>
</tr>
<tr>
<td>blank</td>
<td>508</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>control</td>
<td>1331</td>
<td>823</td>
<td>1.504</td>
</tr>
<tr>
<td>-NADPH</td>
<td>848</td>
<td>340</td>
<td>0.622</td>
</tr>
</tbody>
</table>

$^a$ The radioactivity found in the final aliquot (average of duplicates).

$^b$ The blanks contained 1 ml boiling enzyme suspension.

$^c$ The controls contained the complete reaction mixture as described in 'methods'.
TABLE II

The effect of varied levels of the NADPH-generating system cofactors on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vitro.

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>DPM</th>
<th>DPM MINUS BLANK</th>
<th>IRREVERSIBLY BOUND $^3$H-BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>590</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>control</td>
<td>1571</td>
<td>981</td>
<td>1.793</td>
</tr>
<tr>
<td>5 x glucose-6-phosphate</td>
<td>1619</td>
<td>1029</td>
<td>1.881</td>
</tr>
<tr>
<td>5 x glucose-6-phosphate dehydrogenase</td>
<td>1527</td>
<td>937</td>
<td>1.713</td>
</tr>
<tr>
<td>5 x NADP +</td>
<td>1535</td>
<td>945</td>
<td>1.727</td>
</tr>
<tr>
<td>5 x all of the above</td>
<td>1275</td>
<td>685</td>
<td>1.258</td>
</tr>
</tbody>
</table>

$^a$ The radioactivity found in the final aliquot (average of duplicates).
$^b$ The blanks contained 1 ml boiled enzyme suspension.
$^c$ The controls contained the complete reaction mixture as described in 'methods'.
EFFECT OF IN VITRO ADDITIONS ON THE IRREVERSIBLE BINDING OF $^3$H-BP

The effect of methadone on the irreversible binding of $^3$H-BP in vitro is shown in figure 9. Methadone concentrations of $10^{-4}$M, $10^{-3}$M and $10^{-2}$M decreased the degree of binding from control by 40%, 60% and 43%, respectively. Lower concentrations had no effect.

SKF 525-A at concentrations of $10^{-5}$M and $10^{-4}$M decreased the level of binding from control by 26% and 37%, respectively, as observed in figure 9. Again, lower concentrations had no effect.

Glutathione added to the reaction mixture at a concentration of $10^{-2}$M decreased the irreversible binding of $^3$H-BP from control by 27% as shown in figure 10. At concentrations lower than $10^{-2}$M glutathione had no effect.

Figure 8 shows that cysteine exhibits an inhibition of the irreversible binding of $^3$H-BP from control by 53% at a concentration of $10^{-2}$M. Concentrations of cysteine in the reaction mixture below $10^{-2}$M had no effect on the control level of binding.

$3^1$MC added to the reaction mixture also inhibited the irreversible binding of $^3$H-BP. Figure 12 shows the degree of inhibition from control at $10^{-5}$M, $10^{-4}$M and $10^{-3}$M of $3^1$MC. The percent inhibition at these doses are 27%, 44% and 44%, respectively. It should be noted that none of the compounds used in vitro, at the highest concentration used, altered the level of non-specific binding of $^3$H-BP. The inhibition is the decrease in the enzyme-mediated binding of $^3$H-BP.

RELATIONSHIP BETWEEN IRREVERSIBLE BINDING IN VIVO AND DOSE OF BP

$^3$H-BP (0.139, 1.25, 5.93 or 12.5 μmole i.p.; 125 μCi) in 0.5 ml corn oil was administered to normal rats. The animals were killed 24 hours later and the livers examined for irreversibly bound radioactivity. As shown in figure 13,
Figure 8. The effect of methadone on the irreversible binding of $^3$H-benzo a pyrene ($^3$H-BP) in vitro. Reaction tubes containing $^3$H-BP (1.015 µmole; 2.221 x $10^6$ dpm), the NADPH - generating system, the 10,000 x g supernatant equivalent to a 0.05 gram of liver and various concentrations of methadone were incubated at 37°C for 50 min. Blanks contained boiled enzyme. Values are corrected for non-specific binding and are the averages of 6 tubes (3 separate experiments). Asterisks indicate significant difference from control at p<0.05 (n=6).
Figure 9. The effect of SKF 525-A on the irreversible binding of $^{3}$H-benzo[a]pyrene ($^{3}$H-BP) in vitro. Reaction tubes containing $^{3}$H-BP (1.015 μmole; 2.221 x $10^6$ dpm), the NADPH-generating system, the 10,000 x g supernatant equivalent to 0.05 gram of liver and various concentrations of SKF 525-A were incubated at 37°C for 50 min. Blanks contained boiled enzyme. Values are corrected for non-specific binding and are the averages of 6 tubes (3 separate experiments). Asterisks indicate significant difference from control at $p<0.05$ (n=6).
$^{3}\text{H-BP BOUND}$
nmoles/gram moist weight of liver/min ± s.e.m.
Figure 10. The effect of glutathione on the irreversible binding of 3H-benzo[a]pyrene (3H-BP) in vitro. Reaction tubes containing 3H-BP (1.015 μ mole; 2.221 x 10^6 dpm), the NADPH-generating system, the 10,000 x g supernatant equivalent to 0.05 gram of liver and various concentrations of glutathione were incubated at 37°C for 50 mins. Blanks contained boiled enzyme. Values are corrected for non-specific binding and are the averages of 6 tubes (3 separate experiments). Asterisks indicate significant difference from control at p<0.05 (n=6).
$^{3}$H-BP BOUND
nmoles/gram wet weight of liver/min ± s.e.m.
Figure B1. The effect of cysteine on the irreversible binding of 3H-benzo[a]pyrene (3H-BP) in vitro. Reaction tubes containing 3H-BP (1.015 µmole; 2.221 x 10^6 dpm), the NADPH-generating system, the 10,000 x g supernatant equivalent to 0.05 gram of liver and various concentrations of cysteine were incubated at 37°C for 50 mins. Blanks contained boiled enzyme. Values are corrected for non-specific binding and are the averages of 6 tubes (3 separate experiments). Asterisks indicate significant difference from control at p<0.05 (n=6).
Figure 112. The effect of 3-methylcholanthrene (3-MC) on the irreversible binding of 3H-benzo[a]pyrene (3H-BP) in vitro. Reaction tubes containing 3H-BP (1.015 umole; 2.221 x 10^6 dpm), the NADPH-generating system, the 10,000 x g supernatant equivalent to 0.05 gram of liver and various concentrations of 3-MC were incubated at 37°C for 50 mins. Blanks contained boiled enzyme. Values are corrected for non-specific binding and are the averages of 6 tubes (3 separate experiments). Asterisks indicate significant difference from control at p<0.05 (n=6).
$^3$H-BP BOUND

nmoles/gram wet weight of liver/min ± s.e.m.
the degree of binding to liver tissue, expressed on a per mg. protein basis, was linear over the entire dose range studied. The degree of binding to liver tissue expressed on a per gram wet weight of liver basis was also linear over the entire dose range studied, as observed in figure 14. A second population of animals also showed a linear dose-binding relationship which was markedly lower than the first as shown in figures 15 and 16. The second population represented 25.6% (n=10) of the total number of animals used for the dose binding study (n=39). The correlation coefficients for all 4 curves were greater than 0.999. It was found that the variation in animal weight (200 - 300gm) had no effect on the degree of irreversible binding of $^3$H-BP. The dose of $^3$H-BP used in all subsequent in vivo studies was 1.25 μmole.

**Irreversible Binding in Vivo with Time**

After the administration of $^3$H-BP (1.25 μmole i.p.; 125 μCi) in 0.5 ml corn oil to normal rats, the amount of irreversibly bound radiolabelled material was determined at various time intervals, as shown in figures 17 and 18 (tap water controls). The irreversible binding increased to a maximum of about 400 pmoles/gram wet weight of liver or 2.74 pmoles/mg. protein by 18 hours and fell to about 200 pmoles/gram wet weight of liver or 1.25 pmoles/mg. protein by 48 hours. A second population of rats showed a completely different time-binding relationship, as observed in figures 19 and 20 (tap water controls). At 1½ hours the irreversible binding was at a level of almost 500 pmoles/gram wet weight of liver or 3.5 pmoles/mg. protein and fell to about 60 pmoles/gram wet weight of liver or 0.4 pmoles/mg. protein by 24 hours. This second population represented 15.4% (n=6) of the total number of animals used for the time-binding study (n=39).
Figure 3B. The effect of dose on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) to total rat liver protein and nucleic acids in vivo expressed as pmoles bound/mg. protein. Rats injected with various doses of $^3$H-BP administration and their livers examined for irreversible binding. The correlation coefficient of the regression line was 0.999.
$^{3} \text{H-BP BOUND}$

pmoles/mg protein$\pm$ s.e.m. (n)

dose of $^{3} \text{H-BP}$ (pmoles)
Figure 14. The effect of dose on the irreversible binding of $^{3}$H-benzo[a]pyrene ($^{3}$H-BP) to rat liver macromolecules in vivo expressed as pmoles/bound/gram wet weight of liver. Rats injected with various doses of $^{3}$H-BP in 0.5 ml: corn oil were sacrificed 24 hours after $^{3}$H-BP administration and their livers examined for irreversible binding. The correlation coefficient of the regression line was 0.999.
$^3\text{H-BP Bound}$

pmoles/gram wet weight of liver $\pm$ s.e.m. (n)
Figure 15. The dose vs. irreversible binding curve of $^{3}H$-benzo[a]pyrene ($^{3}H$-BP) in the second population of rats expressed as pmoles bound/mg protein. Rats injected with various doses of $^{3}H$-BP administration and their livers examined for irreversible binding. The correlation coefficient of the regression line was 0.999.
\[ {^3H-BP \text{ BOUND}} \]

\[ \text{pmoles/mg protein ± s.e.m. (n)} \]
Figure 16. The dose vs. irreversible binding curve of $^{3}$H-benzo[a]pyrene ($^{3}$H-BP) in the second population of rats expressed as pmoles bound/gram wet weight of liver. Rats injected with various doses of $^{3}$H-BP in 0.5 ml corn oil were sacrificed 24 hours after $^{3}$H-BP administration and their livers examined for irreversible binding. The correlation coefficient of the regression line was 0.999.
$^{3}$H-BP BOUND

pmoles/gram wet weight of liver ± s.e.m. (n)
Figure 17. The effect of methadone treatment on the irreversible binding of 3H-benzo[a]pyrene (3H-BP) in vivo expressed as pmoles bound/gram wet weight of liver. Rats injected with 3H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil were sacrificed at various times after 3H-BP administration.

--- tap water controls
--- methadone treated

No significant difference from control was seen at any of the times studied (student's t-test, level of significance at p<0.05).
Time after $^3$H-BP administration (hours)

$^3$H-BP BOUND
pmoles/gram wet weight of liver±s.e.m. (n)

- Axis labels and data points indicate a study tracking the binding of $^3$H-BP over time.
Figure 18. The effect of methadone treatment on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) \textit{in vivo} expressed as pmoles bound/mg protein. Rats injected with $^3$H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil were sacrificed at various times after $^3$H-BP administration.

\begin{center}
\includegraphics[width=\textwidth]{figure18}
\end{center}

No significant difference from control was seen at any of the times studied (student's t-test, level of significance at $p<0.05$).
$\text{H-BP BOUND}$

$p\text{moles/mg protein ± s.e.m.}(n)$

time after $\text{H-BP administration (hours)}$
Figure 19. The effect of methadone treatment on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vivo in the second population of rats expressed as pmoles bound/gram wet weight of liver. Rats injected with $^3$H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil were sacrificed at various times after $^3$H-BP administration.

- - - - - - tap water controls

--- methadone treated
Figure 20. The effect of methadone treatment on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vivo in the second population of rats expressed as pmoles bound/mg protein. Rats injected with $^3$H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil were sacrificed at various times after $^3$H-BP administration.

- - - - - tap water controls

- - - - - methadone treated
EFFECT OF PRETREATMENTS ON THE IRREVERSIBLE BINDING OF $^{3}$H-BP IN VIVO

Methadone was added to the drinking water of a series of rats in the following amounts: 0.25 mg/ml for 1 week, 0.50 mg/ml the second week, 0.75 mg/ml the third week, and 1.0 mg/ml for the fourth week. The dose of methadone ingested per animal averaged 50, 80, 95 and 115 mg/kg/day during the four weeks. No evidence of withdrawal symptoms, such as acute loss of weight or wet shakes was observed. This treatment, at a similar dose schedule to the one above, has previously been shown to increase epoxide hydrase activity in male rats to 212% of controls with no change in aryl hydrocarbon hydroxylase activity (66). Paired groups of animals treated with methadone as above showed induction of EH with no changes in AHH activity throughout this study as monitored in this laboratory.

$^{3}$H-BP (1.25 μmole i.p.; 125 μCi) in 0.5 ml corn oil was administered to the methadone pretreated animals and they were sacrificed at either 3, 6, 12, 18, 24 or 48 hours after $^{3}$H-BP. The degree of irreversible binding in the methadone pretreated animals was not significantly different from the tap water controls at any of the times stated, as shown in figures 17 and 18 (methadone treated).

A second population of animals pretreated with methadone had a time-binding curve markedly different from the first, as observed in figures 19 and 20 (methadone treated). This second population of animals represented 27.5% (n=14) of all the animals pretreated with methadone (n=51).

Groups of 4 animals were pretreated with 3-MC (20 mg/kg i.p. for 2 consecutive days) in corn oil, administered $^{3}$H-BP (1.25 μmole i.p.; 125 μCi) in 0.5 ml corn oil 24 hours after the last dose of 3-MC and sacrificed at various times after $^{3}$H-BP. Control animals received corn oil without 3-MC. Figures 21 and 22 show the time-binding curves for these two sets of animals. The irreversible binding in rats pretreated with corn oil rose to a level of 275 pmoles/gram wet weight of liver or 2.1 pmoles/mg protein by 13 hours and remained at about that level to 42 hours after $^{3}$H-BP administration. In rats
Figure 21. The effect of corn oil and 3-methylcholanthrene (3-MC) treatments on the irreversible binding of $^3$H-benzol[a]pyrene ($^3$H-BP) in vivo expressed as pmoles bound/gram wet weight of liver. Rats injected with $^3$H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil were sacrificed at various times after $^3$H-BP administration.

- O- corn oil treated
- - - - - 3-MC treated

Asterisks indicate significant difference from corn oil treated animals at $p<0.05$. 
Figure 22. The effect of corn oil and 3-methylcholanthrene (3-MC) treatments on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vivo expressed as pmoles bound/mg protein. Rats injected with $^3$H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil were sacrificed at various times after $^3$H-BP administration.

- O - corn oil treated
- ---- 3-MC treated

Asterisks indicate significant difference from corn oil treated animals at p<0.05.
pretreated with 3-MC in corn oil, irreversibly bound $^3$H-BP reached a level of 200 pmoles/gram wet weight of liver or 1.5 pmoles/mg protein by 24 hours and maintained that level through to 42 hours. The level of irreversible binding in the 3-MC treated rats was significantly lower than that of the corn oil treated rats at 8, 13, 24 and 42 hours after $^3$H-BP. This represented a 30% decrease from the control levels at each of the times previously mentioned.

The time-binding curve of the corn oil treated animals was both qualitatively and quantitatively different from that of the tap-water control animals. The maximum level of irreversible binding observed in the corn oil treated animals was 72.5% of the highest level observed in the tap water control animals. A decrease to 50% of the maximum level by 48 hours was observed in the tap water animals while the maximum level was essentially maintained to 42 hours in the corn oil treated rats.

A second population of corn oil and 3-MC treated rats displayed time-binding relationships shown in figures 23 and 24. This second group represented 12.1% (n=33) and 22.2% (n=4) of the total number of 3-MC (n=33) and corn oil (n=18) treated rats, respectively. The binding was highest at 1 hour and fell to 15% of this value by 42 hours.

One group of animals pretreated with 3-MC (20 mg/kg i.p. for 2 consecutive days) in corn oil and another with corn oil alone were administered $^3$H-BP (1.25 μmole i.p.; 125 μCi) in 0.5 ml corn oil 48 hours after the last 3-MC or corn oil injection. Figure 25 shows the degree of binding at 24 hours after $^3$H-BP in animals injected with this compound at either 24 or 48 hours after the last dose of 3-MC. There was no statistical difference in the degree of irreversible binding between the two corn oil treated groups ($^3$H-BP 24 hours and 48 hours after the last dose of corn oil). There was also no statistical difference between the two 3-MC treated groups ($^3$H-BP 24 hours and 48 hours after the last dose of 3-MC). However the 3-MC treatment decreased significantly
the binding by 25% and 23% of the corn oil control levels in animals administered $^{3}$H-BP 24 and 48 hours after 3-MC, respectively.

Figure 26 shows the degree of irreversible binding observed in 30% (n=6) of the rats that were administered either corn oil or 3-MC and sacrificed 24 hours after $^{3}$H-BP (n=20). These animals exhibited markedly lower binding levels than animals in the first population.

SKF 525-A (35 mg/kg, 50 mg/kg or 75 mg/kg i.p.) in saline was administered to rats, and control animals were injected with saline alone. $^{3}$H-BP (1.25 μmole i.p.; 125 μCi) in 0.5 ml corn oil was administered 3 hours after SKF 525-A and some animals sacrificed at 3 hours and others at 24 hours after the $^{3}$H-BP. The effect of SKF 525-A on the level of irreversible binding is shown in figure 27. SKF 525-A at 35 mg/kg had no effect on the level of binding at 3 and 24 hours after $^{3}$H-BP as compared to the saline controls. However, SKF 525-A at 50 mg/kg and at 75 mg/kg decreased the binding from control at 3 hours after $^{3}$H-BP by 27% and 31%, respectively and at 24 hours after $^{3}$H-BP by 29% and 34%, respectively. There was no statistical difference in the levels of binding between the saline pretreated and tap water control animals at either 3 or 24 hours after $^{3}$H-BP.

A second population of animals displayed levels of binding shown in figure 27. About 12% (n=7) of all animals used in the SKF 525-A study (n=58) fell into the second population. These levels were markedly lower than the respective levels in first population animals.

Table I11 shows the effect of cysteine and diethyl maleate treatments on the irreversible binding of $^{3}$H-BP. Cysteine (150 mg/kg) in saline administered i.p. 5 minutes before $^{3}$H-BP produced no significant change in binding at either 3 or 24 hours after $^{3}$H-BP administration. The saline control values were the same as the saline and tap water controls previously mentioned (Figures 17 and 27). Diethyl maleate (0.6 ml/kg) in corn oil administered i.p. 30 minutes before $^{3}$H-BP also produced no significant change in binding at 24 hours after $^{3}$H-BP administration. Again, the corn oil control values were the same as
Figure 23. The effect of corn oil and 3-methylcholanthrene (3-MC) treatments on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vivo in the second population of rats expressed as pmoles bound/gram wet weight of liver. Rats injected with $^3$H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil were sacrificed at various times after $^3$H-BP administration.

--- corn oil treated

--- 3-MC treated
Figure 24. The effect of corn oil and 3-methylcholanthrene (3-MC) treatments on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vivo in the second population of rats expressed as pmoles bound/mg protein. Rats injected with $^3$H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil were sacrificed at various times after $^3$H-BP administration.

- - - - corn oil treatment
- - - - 3-MC treatment
Figure 25. A comparison of the effects of corn oil and 3-methylcholanthrene (3-MC) treatments on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vivo in animals injected with the compound at either 24 or 48 hours after the last dose of either corn oil or 3-MC. All rats received $^3$H-BP (1.25 μmole; 125 μCi) in 0.5 ml. corn oil and were sacrificed 24 hours later.

A and B. Rats receiving $^3$H-BP 24 hours after the last dose of corn oil or 3-MC.

C and D. Rats receiving $^3$H-BP 48 hours after the last dose of corn oil or 3-MC.

Asterisks indicate significant difference from corn oil treated animals at p<0.05.
$^{3}$H-BP BOUND
pmoles/gram wet weight of liver ± s.e.m. (n)

- A: Corn oil, 3-MC
- B: Corn oil, 3-MC
- C: Corn oil, 3-MC
- D: Corn oil, 3-MC

Values are shown for different treatments and comparisons are indicated with stars (*) for significant differences.
A comparison of the effects of corn oil and 3-methylcholanthrene (3-MC) treatments on the irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vivo in the second population of rats injected with the compound at either 24 or 48 hours after the last dose of either corn oil or 3-MC.

All rats received $^3$H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil and were sacrificed 24 hours later.

A and B. Rats receiving $^3$H-BP 24 hours after the last dose of corn oil or 3-MC.

C and D. Rats receiving $^3$H-BP 48 hours after the last dose of corn oil or 3-MC.
$^3$H-BP BOUND
pmoles/gram wet weight of liver ± s.e.m. (n)

- A: Corn 3-MC
- B: Corn 3-MC
- C: 3-MC
- D: 3-MC

pmoles/mg protein (SEM)
Figure 27: The effect of SKF 525-A treatment on the irreversible binding of 3H-benzo[a]pyrene (3H-BP) *in vivo*. All animals were injected with 3H-BP (1.25 μmole; 125 μCi) in 0.5 ml corn oil 3 hours after SKF 525-A or saline.

A. Irreversible binding in rats sacrificed 3 hours after 3H-BP.
B. Irreversible binding in rats sacrificed 24 hours after 3H-BP.
C. Irreversible binding in the second population of rats sacrificed 24 hours after 3H-BP.

Asterisks indicate significant difference from saline treated animals at p<0.05.
previously reported (Figures 18 and 22). Table III also shows the level of binding obtained in 2nd population rats. About 13% (n=2) of the animals used in the cysteine and diethyl maleate study (n=16) fell into the 2nd population.

Throughout all the studies combined, the total percent of animals falling into the second population was 19% (46 out of 250 animals).
TABLE III

The effect of cysteine and diethyl maleate treatments on the degree of irreversible binding of $^3$H-benzo[a]pyrene ($^3$H-BP) in vivo.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>IRREVERSIBLY BOUND $^3$H-BP$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmoles/gram wet weight of liver</td>
</tr>
<tr>
<td>saline control$^a$</td>
<td>329.5 ± 14.96 (4)</td>
</tr>
<tr>
<td>cysteine$^a$</td>
<td>376.3 ± 53.83 (3)$^n.s.$</td>
</tr>
<tr>
<td>corn oil control$^a$</td>
<td>97 (1)$^b$</td>
</tr>
<tr>
<td>diethyl maleate$^a$</td>
<td>275.7 ± 15.24 (3)</td>
</tr>
<tr>
<td>saline control$^c$</td>
<td>212.9 ± 20.7 (4)</td>
</tr>
<tr>
<td>cysteine$^c$</td>
<td>260.6 ± 9.37 (3)$^n.s.$</td>
</tr>
</tbody>
</table>

$^a$ The data represents the mean ± S.E.M. (n) of n rats sacrificed 24 hours after $^3$H-BP administration (1.25 μmole; 125 μCi) in 0.5 ml corn oil.

$^b$ Values obtained in the second population of rats.

$^c$ The data represents the mean ± S.E.M. (n) of n rats sacrificed 3 hours after $^3$H-BP administration (1.25 μmole; 125 μCi) in 0.5 ml corn oil.

$n.s$: Mean not significantly different from control at $p<0.05$. 
DISCUSSION

The present study was carried out to determine the roles of AHH, EH, and glutathione on the irreversible binding of BP to total liver protein and nucleic acids in vivo. When $^3$H-BP was injected i.p. into male Wistar rats, a small amount of the compound was bound irreversibly to liver macromolecules. The degree of irreversible binding was found to be dependent on both the dose of BP administered (Figures 13 and 14) and the time after its injection (Figures 17 and 18, tapwater). The results show that pretreating rats with SKF 525-A significantly decreased the level of irreversibly bound BP from control (Figure 27). In addition, pretreating rats with 3-MC also significantly decreased the level of irreversibly bound BP from control (Figures 21 and 22). However, oral methadone pretreatment failed to alter the level of binding to liver macromolecules (Figures 17 and 18, methadone), and neither cysteine nor diethyl maleate pretreatment altered the level of irreversibly bound BP from control (Table III).

In the present study irreversible binding was characterized as follows. The fraction of the administered dose of $^3$H-BP which could not be removed from the TCA precipitated liver homogenate by repetitive solvent extraction was termed irreversibly bound BP. The covalent nature of the BP-macromolecular complexes has been well established (79) and numerous authors have referred to this non-extractable radioactivity as covalently bound (80). To further establish the irreversibility of the unextractable BP and the efficacy of the extraction technique, $^3$H-BP was added to a typical rat liver homogenate either before or after TCA precipitation, as described in Methods. The amount of radioactivity added corresponded to that found in liver homogenates, of corn oil treated rats sacrificed 13 hours after $^3$H-BP, that yielded 276 pmoles of $^3$H-BP/gram wet weight of liver (Figure 21). After the extraction techniques
the radioactivity remaining corresponded to 16 pmoles of \(^3\text{H}\)-BP/gram wet weight of liver when \(^3\text{H}\)-BP was added after TCA precipitation and 8 pmoles of \(^3\text{H}\)-BP/gram wet weight of liver when \(^3\text{H}\)-BP was added before TCA precipitation (Figure 4). These values corresponded to about 5% and 3% of the 276 pmoles of \(^3\text{H}\)-BP bound/gram wet weight of liver, respectively. It was also found that in rats sacrificed immediately after \(^3\text{H}\)-BP administration, only 3 pmoles of \(^3\text{H}\)-BP/gram wet weight of liver was irreversibly bound (Figure 4). Therefore, the efficacy of the extraction techniques was established and thus any radioactivity that could not be removed by these methods was referred to as irreversibly bound.

Prodi and coworkers (81) carried out a study showing the level of binding of BP with time after its i.p. injection into female Wistar rats. The dose injected was 1.25 \(\mu\)mole and they observed the binding of BP at 22, 70, and 168 hours after its administration. The binding to total hepatic protein and nucleic acids was found to be highest at 22 hours after BP, falling to 70% and 37% of maximum by 70 and 168 hours, respectively. They did not report levels of binding at any time less than 22 hours. Jollow and coworkers (75) showed the covalent binding of acetaminophen, a potential hepatotoxin, to liver protein and nucleic acids, with time after its i.p. administration to mice. The binding rose sharply to a maximum by 5 hours and fell to 25% of this value by 24 hours. He also showed that the greatest concentration of acetaminophen in the liver was reached in 2 hours. Therefore, the maximum level of binding occurred after the highest concentration of acetaminophen was reached in the liver. Delwaide (82) showed that BP injected i.p. into rats reached the maximum levels in the liver by 4 to 6 hours, depending on the amount of BP administered. The present study showed the greatest level of binding to occur at 12-18 hours after BP administration, and this fell to 60% of maximum by 48 hours (Figures 17 and 18).
Corn oil treatment was found to decrease both the maximum level of BP binding and the rate by which it reached this level. It was also observed that this highest level was essentially maintained to 42 hours (Figures 21 and 22, corn oil). The effect of corn oil on the binding of BP at 24 hours after its administration was the same when corn oil was administered at either 3, 24 or 48 hours before BP (Table III and Figure 25, corn oil). It appeared that corn oil had an effect on the absorption of BP from the i.p. cavity. Therefore it was essential that all animals pretreated with agents that were dissolved in corn oil had animals that were treated with the corn oil vehicle alone as their controls. Saline pretreatment was found not to alter the binding of BP.

The degree of binding was found to be linearly dependent on the dose of BP between the range of 0.125 and 12.5 μmoles (Figures 13 and 14). This would imply that the enzymatic pathways involving BP metabolism were not saturated. The dose chosen for the in vivo experiments was 1.25 μmole. The binding of BP was expressed on a per mg protein and per gram wet weight of liver basis for the methadone and 3-MC experiments. This was carried out since agents which induce microsomal enzyme activity are sometimes known to induce microsomal protein and total liver protein (83). In all of the experiments performed the protein content of the extracted pellet did not differ for any treatment.

The enzyme responsible for the formation of the irreversibly bound intermediates of BP, the BP epoxides, is a cytochrome P-450 mixed function oxidase, namely BP hydroxylase (39). The decrease in the irreversible binding of BP by SKF 525-A and by 3-MC could be due to their inhibition of BP hydroxylase, thereby decreasing the amount of epoxides produced. SKF 525-A has been shown to inhibit rat hepatic AHH when administered i.p. before sacrifice (74) and when added to the incubation tube (84). 3-MC is also a substrate for AHH (85)
and its presence in the liver could inhibit the metabolism of BP. Thus, the irreversible binding of BP to liver macromolecules was assayed in vitro and the effects of these agents on the in vitro binding of BP was studied.

In the present study, the assay was carried out using the 10,000xg liver supernatant and the NADPH-generating system. After incubation, the TCA-precipitated protein was washed extensively and the degree of irreversible binding quantitated. There was a significant amount of BP bound to the liver macromolecules when boiled enzyme blanks were used. Using active enzyme produced an additional amount of binding. This increase could be reduced by either the deletion of the NADPH-generating system or incubation under N₂ atmosphere. Therefore, the enzymatic nature and the cytochrome P-450 dependency of this binding was established. The non-enzymatic binding, which was not influenced by N₂ or NADPH, was subtracted from the total binding to yield enzymatic binding. Non-enzymatic binding has been discussed in detail by Grilli (86). The enzymatic binding was shown to be linear with incubation time (Figure 5) and protein content (Figure 6) for our assay conditions.

All the drugs tested decreased the enzymatic binding, but not the non-enzymatic binding of BP to liver macromolecules in vitro. SKF 525-A, at a concentration of 10⁻⁵M, produced a significant decrease in the binding of BP (Figure 9). It has been reported that the SKF 525-A concentration in the liver within 2 hours after i.p. injection of 50 mg/kg of the compound may be roughly estimated to be in the range of 10-65 μg/gm or 0.3 to 1.7 x 10⁻⁴ M. It was also reported that after the i.p. injection of 80 mg/kg of SKF 525-A to rats, the concentration in the liver was 68 μg/gm or 1.8 x 10⁻⁴ M 1 hour after the injection and the concentration was found to decrease as a function of time (84). It was shown that SKF 525-A at 35 mg/kg i.p. 3 hours before sacrifice decreased significantly the activity of AHH in vitro (74). In the
present study doses of 50 mg/kg and 75 mg/kg 3 hours before BP were needed to significantly decrease the irreversible binding from control (Figure 27). Also, it was found that SKF 525-A at 35 mg/kg i.p. did not alter significantly the degree of binding. Thus, a dose of 35 mg/kg i.p. apparently did not produce sufficient concentrations of SKF 525-A in the liver to decrease cytochrome P-450 activity sufficiently to inhibit BP binding. In this study doses of at least 50 mg/kg i.p. were necessary to produce inhibition of AHH in vivo and thus reduce the metabolism of BP and its binding to hepatic tissue. The present data indicate that higher levels of SKF 525-A must be obtained in the liver to inhibit BP binding in vivo, in contrast to the lesser amount of SKF 525-A necessary to inhibit the metabolism of BP in vitro. This could suggest that the enzyme responsible for the formation of 3-OH BP in vitro is not the same enzyme responsible for the production of the tissue bound metabolites in vivo, and that the affinities of these enzymes for SKF 525-A are different. It should also be noted that the difference could be a result of the in vitro preparation and assay of the enzyme.

In the present study, 3-MC also decreased significantly the enzymatic binding of BP in vitro (Figure 12). It has been shown that 3-MC will inhibit hepatic AHH in vitro from 3-MC induced rats, but not from untreated rats (87). The concentration of 3-MC used to inhibit AHH in the studies was $1.87 \times 10^{-5}$ M. We found that 3-MC at a concentration of $10^{-5}$ M will inhibit the enzymatic binding of BP in vitro. This decrease in binding appears to oppose the results obtained in the study cited, since we used untreated rats. AHH is studied in vitro by quantitating the formation of 3-OH BP, its major metabolite, from BP. As previously stated, 3-MC will not inhibit the formation of 3-OH BP in untreated animals, but this does not indicate that the other pathways of BP metabolism will be affected, most prominently the 4,5 and 7,8 regions on the BP nucleus. Thomas and Furlong (38) showed that in 3-MC induced rat liver microsomes the formation of the macromolecule-bound species of BP is a major reaction in the in vitro system, occurring at a rate approximately 60% that of the formation of BP phenols. Therefore, it is suggested that the decrease in
binding in vitro could be due to the inhibition of the activation pathways that are responsible for the metabolism of BP to reactive intermediates. BP was administered 24 hours after 3-MC, which was injected i.p. for 2 consecutive days at a dose of 20 mg/kg/day. This treatment produced a 30% decrease in binding from control (Figures 21 and 22). It could be argued that the residual amount of 3-MC present in the liver 24 hours after the last 3-MC injection could be causing a direct inhibition of the metabolic activation of BP. This inhibition was shown in vitro (Figure 12). Therefore, an experiment was carried out in which BP was administered 48 hours after the last 3-MC injection. As shown in figure 25, there was no difference in the percent change from control at 24 hours after BP between the two 3-MC groups. This would suggest that the decrease in binding seen with 3-MC treated animals is not due to the direct inhibition of BP hydroxylase by residual 3-MC.

In the present studies methadone treatment produced no significant change in the degree of BP binding in vivo, but produced a 40% and 60% decrease in binding from control at concentrations in the incubation tube of $10^{-4} M$ and $10^{-3} M$, respectively (Figure 8). Methadone was also found to inhibit BP hydroxylase in vitro at a concentration of $5 \times 10^{-4} M$ (66). However, methadone was administered via the drinking water and the amount of methadone likely to be present in the liver tissue of treated animals is in the range of $10^{-7} M$ (89). It should be noted that methadone was found to increase hepatic epoxide hydratase by 212% in male rats (66) but that the same methadone treatment appeared not to have any influence on the degree of binding of BP in vivo (Figures 18 and 19). 3-MC treatment also induces hepatic epoxide hydratase activity (48) and this could be the cause of the decrease binding of BP in 3-MC treated animals. There is increasing evidence for a single epoxide hydratase that catalyzes the hydration of a wide number of substrates including styrene oxide and BP 4,5-oxide (90).
It has been shown that in 9 human liver samples styrene oxide hydratase activity produced correlations of greater than 0.95 when plotted against the hydration of either the 4,5-, 7,8- or 9,10-epoxide of BP(49). Therefore, it is logical to assume that induction of the hydration of styrene oxide will produce a similar increase in the hydration of any of the BP epoxides. Therefore, it does not seem likely that 3-MC alone could decrease BP binding due to its increase in EH activity without methadone showing a similar effect.

Previous studies have shown that glutathione is essential for the protection of thiol and other nucleophilic groups in animal tissues from a variety of toxic drug metabolites (91). It has been shown that when the dose of acetaminophen is sufficiently large to deplete hepatic glutathione in the form of mercapturic acid conjugates, the metabolite can no longer be detoxified by this pathway and therefore arylates other cellular nucleophiles (67). Similar relationships have been found between hepatic glutathione and hepatic covalent binding of a toxic metabolite of another hepatotoxin, bromobenzene (92). Glutathione conjugates of BP have been identified and glutathione has been shown in vitro to form conjugates with BP epoxides both enzymatically and non-enzymatically (50). In the present study we examined the role of glutathione on the binding of BP both in vivo and in vitro. Glutathione had no effect on the binding of BP to liver macromolecules in vitro until a concentration of $10^{-2}M$ (Figure 10). It was also found that cysteine inhibited the binding of BP at a concentration of $10^{-2}M$ (Figure 11). Neither cysteine nor diethyl maleate pretreatments altered the binding of BP in vivo (Table III). King et al. (70) showed that glutathione at a concentration in the incubation mixture of 3 mM inhibited the binding of BP and BP-7,8-diol to DNA. Both cysteine and glutathione at concentrations of $5 \times 10^{-4}M$ inhibited the binding of acetaminophen to hepatic macromolecules in vitro (93). Thus, glutathione and cysteine are
capable of inhibiting the binding of BP to hepatic macromolecules in vitro, similar to inhibition of acetaminophen binding, at a concentration that is close to the estimated intracellular concentration of glutathione, 3mM (94). The role of glutathione in the binding of BP to hepatic macromolecules appears to be dramatically different than its protective role for certain hepatotoxins, such as acetaminophen. Mitchell showed that covalent binding of acetaminophen to hepatic macromolecules in vivo occurred only with doses of acetaminophen that caused a 70% or more depletion of liver glutathione (67). No significant binding occurred until 70% of the hepatic glutathione was depleted. It was also shown that depleting hepatic glutathione with diethyl maleate produced a marked increase in the binding of acetaminophen with hepatic tissue. Conversely, treatment with cysteine decreased the binding. Cysteine, a glutathione precursor (73), is known to restore the hepatic stores of glutathione (95) when they have been depleted by acetaminophen and it is thought that by this mechanism and by its ability to form conjugates with acetaminophen, cysteine can decrease the binding of acetaminophen metabolites. In the present study, pretreating with diethyl maleate did not alter the binding of BP. Pretreating with cysteine failed to alter the binding of BP, and the binding of BP versus dose, in the range of 0.13 to 12.5 μmole was linear thus indicating the absence of a threshold dose for binding. The cysteine experiment and the low doses of BP used indicated that BP at these doses did not deplete hepatic glutathione. If BP was bound to hepatic macromolecules because it was depleting hepatic glutathione, cysteine treatment should have decreased the binding and diethyl maleate treatment should have increased the binding. Therefore, there appears to be no protective role of glutathione for BP binding by the same mechanism as it protects the liver from acetaminophen active metabolites. The present data suggest that at low doses of BP there is insignificant alteration in glutathione levels and that even after depletion of
70\% of the hepatic glutathione, there is sufficient glutathione present to conjugate with BP. Since glutathione conjugates of BP epoxides are known to exist the presence of glutathione is necessary. But with low doses of carcinogens, the amount necessary would be far less than the amount required to detoxify drugs which are taken in therapeutic quantities.

A second possible mechanism for glutathione should be considered. The controlling factor in the formation of glutathione conjugates of BP could be the GSHT instead of the concentration of hepatic glutathione. GSHT catalyzes the conjugation of glutathione with BP epoxides (50).

3-MC has been shown to increase the conjugation of epoxides by inducing GSHT activity (51). The decrease in binding with 3-MC could be due to the induction of these enzymes. Unfortunately, we do not have, at this time, a compound which selectively induces or inhibits this enzyme and therefore a decrease in BP binding due to an increase in GSHT activity by 3-MC remains a possibility.

The 3-MC induced decrease in BP binding could also be due to an alteration in the pathways in BP metabolism. Zampaglione (96) and coworkers found that 3-MC induced protection from bromobenzene's hepatotoxicity. SKF 525-A also blocked bromobenzene-induced liver necrosis, but it blocked bromobenzene metabolism in vivo. 3-MC did not alter the rate of bromobenzene metabolism in vivo. It was found that there was an increase in bromophenyldihydrodiol, bromocatechol and 2-bromophenol excretion. It was suggested that 3-MC induction may direct bromobenzene metabolism into a comparatively nontoxic pathway. Wiebel (39) showed the metabolism of BP by different forms of cytochrome P-450 from rabbit liver. He found that the relative amounts of BP metabolites formed by various cytochrome preparations and by liver microsomes were dramatically different. He therefore stated that since the mixed function oxidases were
engaged in both the activation and detoxification of PAH's, differences in the relative distribution of the multiple forms of cytochrome P-450 might be a key factor in determining the susceptibility of tissues, individuals, and species to the cytotoxic and carcinogenic action of PAH's. Kinoshita (97), using liver microsomes from control and 3-MC treated rats showed an increase in the overall metabolism of BP with microsomes from 3-MC treated animals, but more importantly, showed a larger increase in metabolism at the non-K-region of BP versus the K-region. Thus, the existence in the liver of various forms of cytochrome P-450, along with the evidence that 3-MC induces a spectrally distinct cytochrome P-448 (40, 58), could suggest that 3-MC alters BP metabolism towards pathways that produce less reactive intermediates, and thereby decrease the degree of binding.

In isolated perfused lungs Cohen (71) showed that 3-MC increased the binding of BP to lung tissue. A dose of 2 nmole BP was instilled intratracheally and the nonrecirculating perfusate was collected. The increase in binding in lung tissue is opposite to the results obtained in the present experiments, which showed a decrease in hepatic binding of BP in 3-MC treated rats. This difference could lie in the dose of BP used in the two separate experiments. It is possible that 2 nmoles administered intratracheally could be saturating the cytochrome P-450 enzyme units at the sites of metabolism. Induction by 3-MC, shown to increase BP-hydroxylase activity by increasing the amount of enzyme present (98) could therefore increase the formation of reactive metabolites in the 3-MC treated rats. In the present experiment, BP is administered i.p. and, at any given time, the amount of BP that passes through the liver, which contains a 20-fold greater quantity of cytochrome P-450 than the lung (99), might not saturate the enzyme sites. Thus, in this case, 3-MC induction would not affect the rate of metabolism, but instead affect the pathways of metabolism, i.e., induce the formation of cytochromes with different site
preferences on the BP molecule that leads to the production of less reactive intermediates.

Furthermore, induction by 3-MC in the lung versus the liver after its i.p. administration has been studied (100). It was shown that lower doses of 3-MC, which produced induction of AHH in the lung, did not affect the activity of this enzyme in the liver. The fold-induction of the enzyme was shown to be much higher in the lung than in the liver. This is further evidence for the existence of different forms of cytochrome P-450 within and amongst various organs and evidence for differences in their relative inducibility.

Throughout the study a second population of animals showed binding of BP that was both qualitatively and quantitatively different from the first. The percentage of animals that fell into this 2nd population was 19% (46 out of 250 animals). There is no mention of this phenomenon in the literature and the causes of this difference is unknown at this point in time. The time-binding curve in this 2nd population of animals (Figures 19 and 20, tap water) is similar to the kinetics of the plasma drug concentration with time after its i.v. administration. Whether this observation of two distinct animal populations is due to a difference in absorption, distribution, or metabolism between the two groups is at present highly speculative. Sex, species and strain variations in drug metabolism are commonly known (101). Certain strains of mice show an increase in hepatic AHH activity with 3-MC treatment, whereas AHH in certain other strains are non-inducible with 3-MC (102). Again a genetic difference between the present study's two groups in their ability to metabolize BP is also speculative.

One possible mechanism to account for the observations found is that the degree of binding of BP to liver macromolecules in vivo after its i.p. administration into rats appears to depend strongly on the relative abundance of the various forms
of cytochrome P-450 present. The decrease in binding with 3-MC treatment could be due to the formation of different cytochromes which alter the metabolism of BP to pathways which produce less reactive intermediates. The decrease in binding after SKF 525-A treatment is in agreement with the evidence that the cytochrome P-450 dependent enzymes are responsible for the activation of BP to reactive intermediates and that these enzymes can be inhibited by SKF 525-A (42). The lack of a role for glutathione regarding the binding of BP is dramatically different from its role in the protection of acetaminophen hepatic binding, and is probably due to a physiological excess of this sulfhydryl compound as compared to the small quantity of polycyclic aromatic hydrocarbon.

In order to clarify which parameters control the activation and binding of BP to cellular macromolecules a number of experiments are necessary. Isolation of the various forms of hepatic and lung cytochrome P-450 and a study of the kinetics of BP metabolism and binding with these enzymes would be of major interest in light of the present experiments. Secondly, repeating the 3-MC experiments in lung tissue could give a better comparison with the studies of Cohen (71). Finally, studies using higher doses of BP to see if the results of treatment are different at doses of BP that saturate the hepatic cytochrome P-450 enzymes would give stronger support to the discussion of the present data.
SUMMARY AND CONCLUSIONS

1. The hepatic irreversible binding of $^3$H-BP \textit{in vivo}, administered i.p., was found to be both dose and time dependent.

2. The degree of binding was shown to be linearly dependent on the dose of BP administered between 0.125 and 12.5 μmole BP.

3. The maximum level of binding occurred at 12 to 18 hours after BP administration and fell to 60% of maximum by 48 hours.

4. SKF 525-A, an agent which inhibits AHH \textit{in vitro} (74), decreased the irreversible binding of BP to total liver protein and nucleic acids \textit{in vivo} to about 70% of control.

5. 3-MC, an agent which induces AHH and the formation of a spectrally distinct form of cytochrome P-450, namely cytochrome P-448 (40, 58), decreased the irreversible binding of BP to liver macromolecules \textit{in vivo} by 30% from control levels.

6. Cysteine, a precursor of glutathione (73), and diethyl maleate, which depletes hepatic glutathione (72), had no significant effect on the level of irreversibly bound BP from control.

7. Methadone, which was shown to selectively increase EH activity (66) also had no effect on the level of BP irreversibly bound to liver macromolecules \textit{in vivo}.

8. Cysteine ($10^{-3}$M), glutathione ($10^{-2}$M), 3-MC ($10^{-5}$M), SKF 525-A ($10^{-5}$M), and methadone ($10^{-3}$M), all significantly decreased the binding of BP \textit{in vitro} to 10,000 x g. liver supernatant macromolecules. Lower concentrations had no significant effect.

9. A second population of animals exhibited binding of BP that was both qualitatively and quantitatively different from the first. The 2nd population represented 19% (46 out of 250) of the total number of animals used in the study.
These results indicate that the \textit{in vivo} irreversible binding of $^3$H-BP to rat hepatic tissue is mainly dependent on the activity of the cytochrome P-450 mixed function oxidase enzyme system. Increased levels of EH, or changes in glutathione levels do not appear rate-controlling.
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