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Drug Metabolism Concepts

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Drug Metabolism Concepts

Donald M. Jerina, EDITOR
The National Institutes of Health

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FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the SERIES parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that in order to save time the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. As a further means of saving time, the papers are not edited or reviewed except by the symposium chairman, who becomes editor of the book. Papers published in the ACS SYMPOSIUM SERIES are original contributions not published elsewhere in whole or major part and include reports of research as well as reviews since symposia may embrace both types of presentation.

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PREFACE

The metabolism of drugs and other environmental chemicals has attracted the attention and concern of scientists whose specialties range from analytical and physical chemistry to toxicology, biology, and ecology. The efforts of enzymologists, biochemists, and pharmacologists have established that the basic components of microsomal drug metabolism consist of a membrane or lipid environment, reductases for electron transport, and a family of heme-containing terminal oxidases at which half of the oxygen molecule is reduced to water and the other half is incorporated into substrate. The mechanism by which dioxygen undergoes two-electron reduction to form water and an "active oxygen" species has fascinated inorganic and organic chemists alike. Rates and pathways of metabolism are critically important to medicinal chemistry, pharmacology, and clinical medicine. Chemically reactive intermediates are of concern to toxicologists and to biologists studying carcinogenicity and provide a special fascination for the chemist in terms of structure-activity relationships. Thus, the metabolism of drugs and other xenobiotics not only effects detoxication by conversion to polar, readily excretable compounds but, in many instances, forms reactive intermediates which are responsible for toxic or carcinogenic effects. The concept that chemical carcinogens induce neoplasia via conversion to reactive metabolites which cause chemical modification of critical cellular molecules has made the field of drug metabolism very relevant to the effects of drugs and environmental pollutants on man and his world.

This volume is the outgrowth of a symposium titled "Recent Advances in the Study of Drug Metabolism." For this symposium, an effort was made to assemble scientists who would represent a broad spectrum of research in drug metabolism. Hopefully, this interdisciplinary collection will prove of value.

The National Institutes of Health
Bethesda, Md.
December 1976

DONALD M. JERINA

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Cytochrome P-450—Its Role in Oxygen Activation for Drug Metabolism

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At this time many of our colleagues are directing their attention (1,2) to evaluating the existence of oxygen on the planet Mars - an effort dictated by the desire to learn whether any life form, as we know it, exists on that planet. What is it that confers on oxygen special properties that make it so critical for the functioning of cellular metabolism. Much has been written on the "fitness of oxygen" (3) pointing to the capability to metabolically reduce atmospheric oxygen to hydrogen peroxide or water by two or four electron transfer processes, respectively. More important to the topic of this symposium is the ability to enzymatically "activate" molecular oxygen, permitting the incorporation of one atom of oxygen into an organic substrate molecule concomitant with the reduction of the other atom of oxygen to water. The enzyme systems responsible for catalyzing these types of reactions (Figure 1) are termed mixed-function oxidases, hydroxylases or oxygenases. In many instances, the introduction of a hydroxyl group to the hydrophobic substrate molecule provides a site for subsequent conjugation with hydrophilic compounds thereby increasing the solubility of the product for its transport and excretion from the organism. Central to the functioning of many mixed-function oxidation reactions is a family of hemoproteins generally classified as cytochromes P-450. The present symposium is directed to further our understanding of how this unique hemoprotein participates in "oxygen activation" and "substrate hydroxylation".

Studies with the perfused rat liver by Thurman and Scholz (4, 5,) as well as Sies, *et al*. (6,7) indicate that approximately 60 percent of cellular respiration by this organ is sensitive to inhibition by Antimycin A - a chemical well recognized as a powerful inhibitor of the mitochondrial respiratory chain. Of the remaining 40 percent of cellular respiration approximately half is sensitive to the inhibitor sodium cyanide. A significant portion

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of the antimycin A insensitive respiration of the liver cell is presumed to occur by oxidative enzymes associated with the endoplasmic reticulum, i.e. the microsomal fraction. A consideration of microsomal electron transfer reactions (Figure 2) reveals the functioning of at least three flavoproteins, two hemoproteins, and an iron containing protein. The flavoproteins function as reduced pyridine nucleotide dehydrogenases and they participate in the transfer of reducing equivalents to cytochrome b_5 from NADH, via the flavoprotein (fp_1), NADH-cytochrome b_5 reductase, or from NADPH by (fp_2), NADPH-cytochrome P-450 reductase (8). Reduced cytochrome b_5 may interact with a recently isolated iron containing protein (9) in the cyanide sensitive desaturation of fatty acyl-Coenzyme A compounds (10,11). The reduced flavoprotein, fp_2, can also undergo oxidation by ferric cytochrome P-450 in reactions to be described below, or, by an as yet poorly understood reaction, this reduced flavoprotein has been postulated (12,13) to react with molecular oxygen giving rise to a superoxide anion for the initiation of lipid peroxidation or heme degradation. A third flavoprotein, fp_3, participates in the oxidation of tertiary amines giving rise to N-oxides as described by Ziegler et al. (14,15). Of interest are recent results reported by Ziegler et al. (16) that this flavoprotein, fp_3, may also function in an oxygen dependent oxidation of sulfhydryl groups for the formation of disulfide bonds.

Cytochrome P-450 has many interesting properties that serve as a challenge to the biochemist concerned with understanding the function of this hemoprotein as it participates in a broad spectrum of oxidative reactions. Its natural environment in most mammalian tissues is the membrane structure called the endoplasmic reticulum; therefore, considerable interest is directed to understanding the influence imposed by a presumed restricted mobility of protein molecules in a mileu of lipid as this pigment interacts with oxygen, substrate molecules, and the flavoprotein electron donor. Further, it requires the skill and perseverance of groups such as those led by Coon et al. (17-19) as well as Lu and Levin (20-22) to isolate and purify various forms of this hemoprotein for physical and chemical characterization. Such studies have revealed that the family of cytochromes P-450 have molecular weights in the range of 46,000 to 52,000 and display a great propensity to aggregate because of their hydrophobic properties. Further, cytochrome P-450 is readily inducible upon treatment of an animal with various drugs or polycyclic hydrocarbons. Although this property is poorly understood it appears to be responsible in part for the long observed phenomenon of drug tolerance (23,24). As will be discussed by Dr. Coon during this symposium (25), cytochrome P-450 can exist in multiple forms with an ever increasing roster of new proteins identified as purification methodology becomes more sophisticated and widely used.

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Initial interest in microsomal mixed-function oxidation reactions occured in the 1950's; such studies were focused on three general areas of biomedical importance. Brodie and his collaborators (26,27) recognized the potential role of hydroxylation reactions in the oxidative transformation of many drugs - hence this reaction system was looked upon as a general mechanism for detoxification of foreign chemicals. In contrast, the Millers and their colleagues (28,29) were concerned with the oxidative conversion of precarcinogenic chemicals, such as polycyclic hydrocarbons, to carcinogens - reactions of great potential harm to the maintenance of the viability of the organism. A third group, the steroid endocrinologists, were actively studying (30, 31) the oxidative processes responsible for the enzymatic conversion of cholesterol to glucocorticoids and mineralocorticoids - products of critical importance for the maintenance of homeostasis of the organism. We now know that the general family of hemoproteins, cytochromes P-450, play pivitol roles in directing the operation of each of these, as well as other similar reactions. Thus this class of hemoproteins has a duality of function (Figure 3) i.e., it may serve as either a panacea or a plague for the cell.

Proposed Cyclic Function of Cytochrome P-450. How do we currently visualize the function of this unique hemoprotein, cytochrome P-450? One postulated scheme (32) illustrating the cyclic pattern of reduction and oxygenation of cytochrome P-450 as it interacts with substrate molecules, electron donors, and oxygen is shown in Figure 4. Briefly, these reactions may be summarized as follows:

A. The ferric hemoprotein (Fe^{+3}) can interact with a molecule of substrate (R) resulting in a complex ($Fe^{+3}\cdot R$) analogous to an enzyme - substrate complex. This interaction can be directly measured since the substrate molecule appears to be closely associated with the heme moiety of cytochrome P-450 resulting in a spectral perturbation measurable by either optical absorbance spectrophotometry or electron paramagnetic resonance spectrometry (33-36). The orientation of the substrate molecule as it sits in proximity of the heme iron, the presumed binding site for oxygen, remains a conjecture although the role of steering groups on the substrate molecule (37) or the influence of the hydrophobic environment of the heme (38) may be considered as possible directing forces.

B. The substrate complex of ferric - cytochrome P-450 ($Fe^{+3}\cdot R$) undergoes reduction to a ferrous cytochrome P-450 - substrate complex ($Fe^{+2}\cdot R$) by electrons originating from NADPH and transferred by the flavoprotein (fp_2), NADPH-cytochrome P-450 reductase. The question of a stimultaneous two electron transfer to cytochrome P-450, as suggested by Coon et al. (39,40), or the existence of two discrete one electron donating steps, as demonstrated for the purified bacterial cytochrome P-450 by Tyson et

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$$2e^- + \text{Substrate (hydrophobic)} + O_2 \xrightarrow[\text{M.F.O.}]{\text{oxygenase}} \text{Product (hydroxylated)} + H_2O$$

Product (hydroxylated) → Conjugation → Excretion

Figure 1. General representation of microsomal-catalyzed oxygenase or mixed-function oxidation reactions

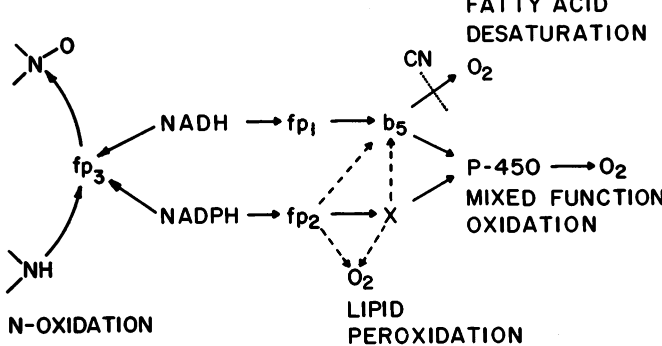

Figure 2. Schematic of microsomal electron transport reactions

A. Active Drug $\xrightarrow{O_2,\, e^-}$ Inactive Drug

B. Precarcinogen $\xrightarrow{O_2,\, e^-}$ Carcinogen

Figure 3. The duality of cytochrome P-450-catalyzed reactions

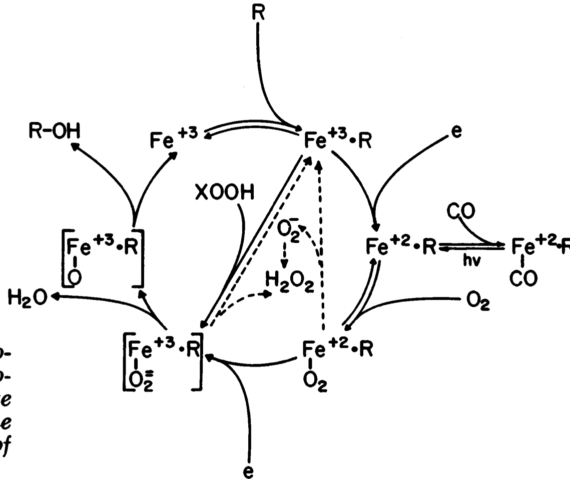

Figure 4. Schematic of the proposed cyclic function of cytochrome P-450. The substrate molecule is designated R. The valence state of the heme iron of cytochrome P-450 is indicated.

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al. (41) as well as Peterson (42), remains as a point of uncertainty. For the present discussion the scheme presented is based on the premise that two separate electron donating reactions occur.

C. Reduced cytochrome P-450 ($Fe^{+2}\cdot R$) can react with carbon monoxide to form a derivative which is readily identifiable spectrophotometrically by an absorbance band maximum at about 450 nm - hence the origin (43) of the name cytochrome P-450. Alternatively, reduced cytochrome P-450 can react with oxygen to form a complex termed (44) oxycytochrome P-450 ($Fe^{+2}\cdot O_2\cdot R$). Knowledge of the chemistry of oxycytochrome P-450 should provide the needed clue to evaluate the first step of "oxygen activation" for hydroxylation reactions; therefore, much effort has been directed to understanding the parameters that influence the generation and subsequent utilization of this intermediate in cytochrome P-450 catalyzed reactions.

D. Oxycytochrome P-450 ($Fe^{+2}\cdot O_2\cdot R$) can presumably dissociate to give a superoxide anion (O_2^-) concomitant with the regeneration of the ferric hemoprotein. The resultant hydrogen peroxide formed by dismutation of the superoxide anion may be a measure of this abortive "uncoupling" (45,46) of cytochrome P-450 function, i.e., a reaction which diverts the ternary complex of oxygen, hemoprotein, and substrate from its role in oxygen activation and subsequent substrate hydroxylation. Alternatively, the complex of oxycytochrome P-450 may undergo further reduction to form the equivalent of a peroxide anion derivative of the substrate bound hemoprotein. Studies (47-50) with the membrane bound cytochrome P-450 of liver microsomes suggests that a donation of a proposed second electron occurs via cytochrome b_5 (51). This conclusion is the basis for explaining the synergistic effect observed during the concomitant oxidation of NADPH and NADH by this system (52).

E. The proposed peroxide anion complex of cytochrome P-450 may undergo protonation and dissociate as hydrogen peroxide or it may rearrange to form an oxene derivative (53) concomitant with the release of water. The existence of oxygen as an oxenoid species is conjecture although studies with organic peroxide supported hydroxylation reactions (54-58) mediated by cytochrome P-450 demonstrate the existence of an EPR species similar to Complex I of peroxidase as an intermediate in the reaction (59). Clearly, more research is needed to firmly establish the presence of this form of oxygen as "active oxygen".

F. Least understood is the mechanism of dissociation of the hydroxylated product and the restoration of the low spin form of ferric cytochrome P-450. In some instances (60) epoxide intermediates exist, such as occurs in those reactions involving polycyclic hydrocarbons (61). In other cases, product adducts result (62-65) which impede the further function of cytochrome P-450 as it participates in substrate hydroxylation reactions.

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Hydrogen Peroxide Formation. The generation of hydrogen peroxide during NADPH oxidation by liver microsomes has been recognized for a number of years (66,67). Little attention was paid to this phenomenon - in large part because of the association of catalase with the microsomal fraction resulting in the breakdown of hydrogen peroxide as rapidly as it was formed. Recent studies (54-58) on the peroxidatic function of cytochrome P-450 as well as the coupled reaction of hydrogen peroxide with catalase for the oxidation of alcohols (68-70) during NADPH oxidation by liver microsomes has stimulated a further consideration of the reactions involved in hydrogen peroxide formation. In addition, an understanding of the mechanism of hydrogen peroxide generation may provide a needed clue for better examining the properties of "activated oxygen" proposed to be a necessary intermediate in cytochrome P-450 catalyzed reactions (71).

As illustrated in Figure 5, the addition of NADPH to a suspension of rat liver microsomes incubated in the presence of sodium azide to inhibit adventitious catalase, results in a stoichiometric reduction of oxygen. Approximately 50 percent of the oxygen reduced can be accounted for as hydrogen peroxide generated during the reaction. Repeated additions of NADPH results in the stepwise formation of equal increments of hydrogen peroxide indicating the ability to additively form this product. The failure to observe a stoichiometric amount of hydrogen peroxide formed to the amount of oxygen reduced or NADPH oxidized has been attributed (72) to the presence of "endogenous substrates" associated with the microsomal fraction which can undergo mixed function oxidation reactions. The nature of the proposed "endogenous substrates" remains to be better defined.

A number of possibilities exist to explain the source of hydrogen peroxide. As proposed in the scheme presented in Figure 4 hydrogen peroxide may arise either from dissociation of oxycytochrome P-450 or from the two electron reduced form of the ternary complex of oxygen, substrate, and cytochrome P-450. Alternatively one must consider that hydrogen peroxide formation may not involve cytochrome P-450, such as by the autoxidation of reduced flavoproteins. In order to firmly establish the role of cytochrome P-450 in hydrogen peroxide generation a series of experiments were carried out to examine the influence of inhibitors of cytochrome P-450 function, such as carbon monoxide or metyrapone, as well as determine the influence of temperature, pH, and the effect of various levels of NADPH on the reaction. In each instance comparative studies were carried out to determine the changes observed in the rate of N-demethylation of ethylmorphine - a substrate recognized to undergo oxidative transformation catalyzed by cytochrome P-450.

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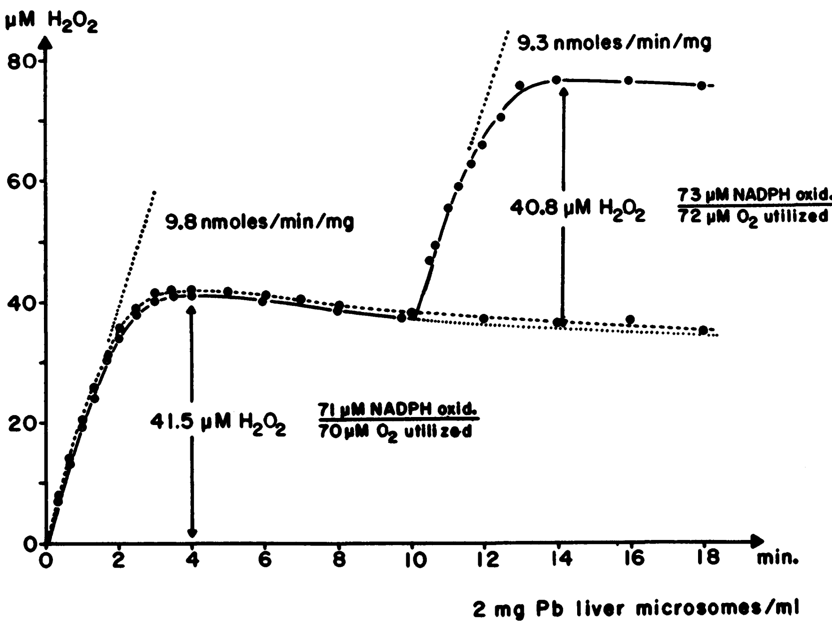

Figure 5. The formation of hydrogen peroxide during NADPH oxidation.
Liver microsomes from phenobarbital-treated rats were incubated at 2 mg of protein per ml in a reaction medium containing 50mM tris-chloride buffer, pH 7.5, 150mM KCl, 10 mM $MgCl_2$, and 1mM NaN_3. At times equal to 0 and 10 min, aliquots of NADPH were added to initiate the reaction. In the experiment shown in dashes, NADPH was added only at 0 time. Samples were removed at the points indicated and after addition to trichloroacetic acid, the concentration of hydrogen peroxide formed was determined colorometrically with potassium thiocyanate and ferrous ammonium sulfate (83). Oxygen use was measured in a comparable series of experiments polarographically, and NADPH oxidation was determined spectrophotometrically.

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Incubation of liver microsomes in a vessel designed to maintain fixed ratios of carbon monoxide to oxygen results in an equivalent increase in inhibition of both hydrogen peroxide formation and ethylmorphine N-demethylation as the concentration of carbon monoxide increases relative to oxygen as shown in Figure 6. The observation that hydrogen peroxide formation is inhibited by carbon monoxide is strong presumptive evidence for the role of cytochrome P-450 in this reaction (73). The fact that the extent of inhibition is effectively identical to that observed for the metabolism of ethylmorphine reinforces this conclusion. However, to date no photochemical action spectrum studies (74,75) have been carried out to confirm the role of cytochrome P-450 in the formation of hydrogen peroxide as has been done for other substrates oxidatively metabolized by this hemoprotein. It is of interest to note that the degree of carbon monoxide inhibition is independent of the presence of NADH where a synergistic (47-50) effect on product formation during the NADPH supported reaction may occur (see below).

Studies with the inhibitor metyrapone (2-methyl-1,2-bis(3-pyridyl)-1-propanone) are more equivocal since hydrogen peroxide formation is maximally inhibited about 50 percent by this compound whereas the metabolism of ethylmorphine is greater than 90 percent inhibited (Figure 7). One possible explanation for this difference relates to the observation that only 50 percent of the cytochrome P-450 associated with liver microsomes from phenobarbital treated rats reacts with metyrapone (76) to form a spectrally identifiable complex. This would imply that all forms of cytochrome P-450 present in liver microsomes can participate in hydrogen peroxide formation whereas a unique metyrapone binding form functions in ethylmorphine N-demethylation reactions. Clearly more experiments will be required to support this hypothesis.

An examination of the influence of varying suboptimal steady state levels of NADPH shows (Figure 8) that the apparent Km's for NADPH required to support the generation of hydrogen peroxide and the N-demethylation of ethylmorphine are identical. Likewise studies on the influence of temperature (Figure 9) on the rates of the two reactions shows an equivalent calculated energy of activation of approximately 20 kilocalories per degree. Thus a wide range of different factors have been examined to establish the similarity of reactions in which cytochrome P-450 serves as the pigment interacting with oxygen for the formation of hydrogen peroxide. The question remains unanswered, however, as to the mechanism of hydrogen peroxide generation, i.e. by dismutation of the superoxide anion dissociating from oxycytochrome P-450 or by protonation of the proposed two electron reduced state equivalent to the peroxide anion complex of cytochrome P-450.

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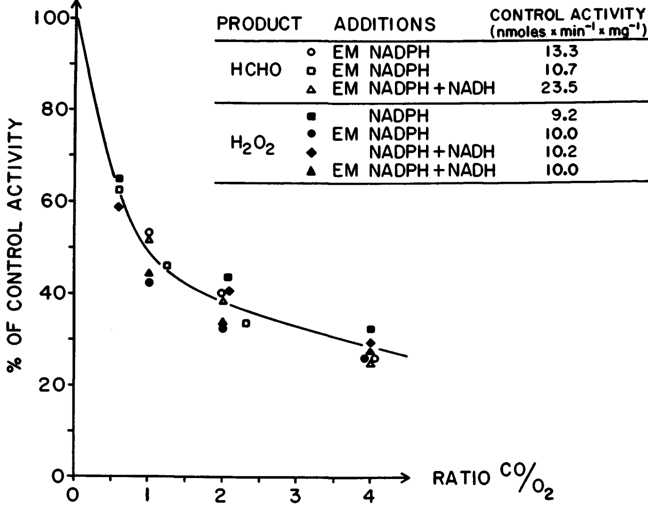

***Figure 6.** The inhibition by carbon monoxide of the generation of hydrogen peroxide and the N-demethylation of ethyl morphine.*

A series of experiments was carried out as described in Figure 5 in a reaction vessel designed to permit equilibration with various gas mixtures of carbon monoxide, oxygen, and nitrogen. The concentration of oxygen was maintained at 20% for all experiments. The reaction mixture was supplemented by adding 5mM sodium isocitrate, 0.5 units of isocitrate dehydrogenase per ml, 2mM of 5'AMP, and 5μM rotenone. The reaction was initiated by adding 200μM NADPH and 200μM NADPH where indicated. Samples were removed every 30 sec for the initial 5 min of the reaction and analyzed for hydrogen peroxide (83) or formaldehyde (84).

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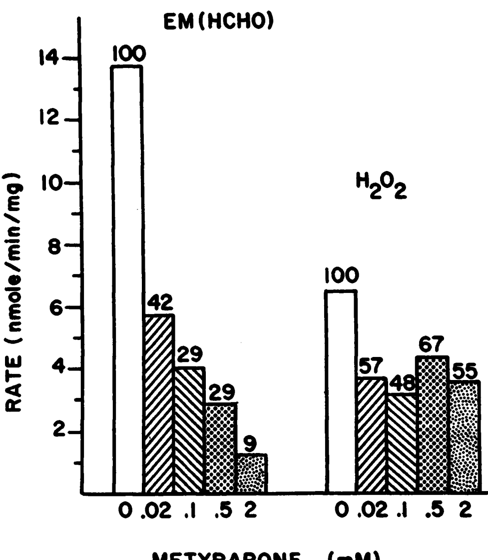

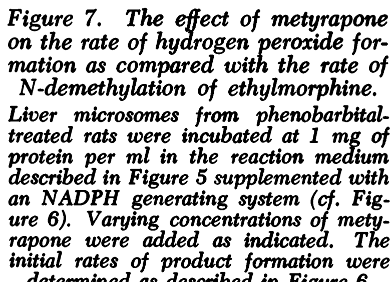
Figure 7. The effect of metyrapone on the rate of hydrogen peroxide formation as compared with the rate of N-demethylation of ethylmorphine.

Liver microsomes from phenobarbital-treated rats were incubated at 1 mg of protein per ml in the reaction medium described in Figure 5 supplemented with an NADPH generating system (cf. Figure 6). Varying concentrations of metyrapone were added as indicated. The initial rates of product formation were determined as described in Figure 6.

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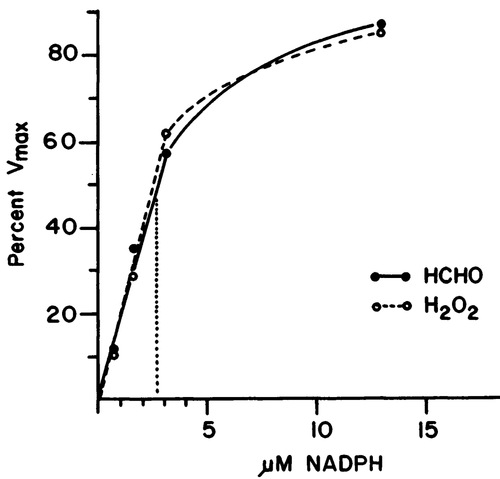

Figure 8. The influence of varying steady state concentrations of NADPH on the rate of formation of hydrogen peroxide and the N-demethylation of ethylmorphine.

Liver microsomes from phenobarbital-treated rats were incubated in a reaction medium as described in Figure 5 supplemented with 5mM sodium isocitrate and 0.5 units of isocitrate dehydrogenase per ml. Where indicated, 5mM ethylmorphine was present. Varying concentrations of NADPH were added to initiate the reaction, and the rate of hydrogen peroxide or formaldehyde formed was determined as described in Figure 6.

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The Influence of Substrates of Cytochrome P-450 on the Generation of Hydrogen Peroxide. Presumably oxycytochrome P-450 serves at a pivitol point in the cyclic function of cytochrome P-450 (cf. Figure 4). This ternary complex of oxygen, substrate and cytochrome P-450 can undergo dissociation to give rise to hydrogen peroxide regenerating the complex of ferric cytochrome P-450 with substrate or it can proceed, by a mechanism as yet unknown, to activate oxygen for insertion into the substrate resulting in the formation of a hydroxylated product and the low spin uncomplexed form of ferric cytochrome P-450. Therefore it was of interest to carry out experiments to evaluate the influence of various substrates of cytochrome P-450, known to undergo enzymatic hydroxylation, and to determine how such substrates influence the rate of hydrogen peroxide formation. As illustrated in Figure 10, the presence of ethylmorphine markedly attenuated the extent of hydrogen peroxide formed when a limiting amount of NADPH is added to initiate the reaction (cf. Figure 5). In addition a small but reproducible inhibition of the initial rate of hydrogen peroxide formation was observed. The decrease in the extent of hydrogen peroxide formation in the presence of ethylmorphine can be attributed in large part to the stimulation of NADPH oxidation observed in the presence of this substrate, i.e. a nearly additive effect of NADPH oxidation occurs when a N-demethylation reaction functions concomitant with hydrogen peroxide formation. Of interest is the observation that a slow but perceptible rate of N-demethylation of ethylmorphine continues after the total oxidation of NADPH. This slow reaction (occurring after 4 minutes in the experiments shown in Figure 10) appears related to a stimulation in the rate of utilization of hydrogen peroxide in the presence of sodium azide. As discussed below, the ability to support the oxidative metabolism of a variety of substrates by hydrogen peroxide, in the absence of reducing equivalents generated from NADPH, directly demonstrates the peroxidatic function of cytochrome P-450.

A further series of experiments were carried out to examine the effect of other substrates on the rate of formation of hydrogen peroxide as summarized in Figure 11. In this case NADPH concentration was maintained by the addition of sodium isocitrate and isocitrate dehydrogenase. In agreement with the experimental results described in Figure 10, the presence of ethylmorphine resulted in approximately a 20 percent inhibition of the rate of hydrogen peroxide formation. In contrast, substrates of cytochrome P-450 such as benzphetamine and hexobarbital caused a marked stimulation in the rate of hydrogen peroxide formation. Ullrich and Diehl (45), Hildebrandt et al (46) and Werringloer et al (70) have described the ability of various compounds to serve as "uncouplers" of cytochrome P-450 function, i.e., chemicals which stimulate the rate of NADPH oxidation and oxygen utilization without a corresponding increase in the rate of substrate hydroxylation. There appear to be two classes of such "uncouplers"; those

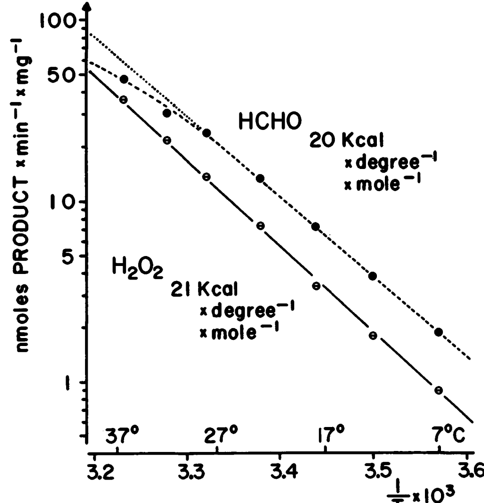

Figure 9. The effect of varying temperatures on the rate of hydrogen peroxide formation and the rate of N-demethylation of ethylmorphine. A series of experiments similar to those described in Figure 6 were carried out at the temperatures indicated.

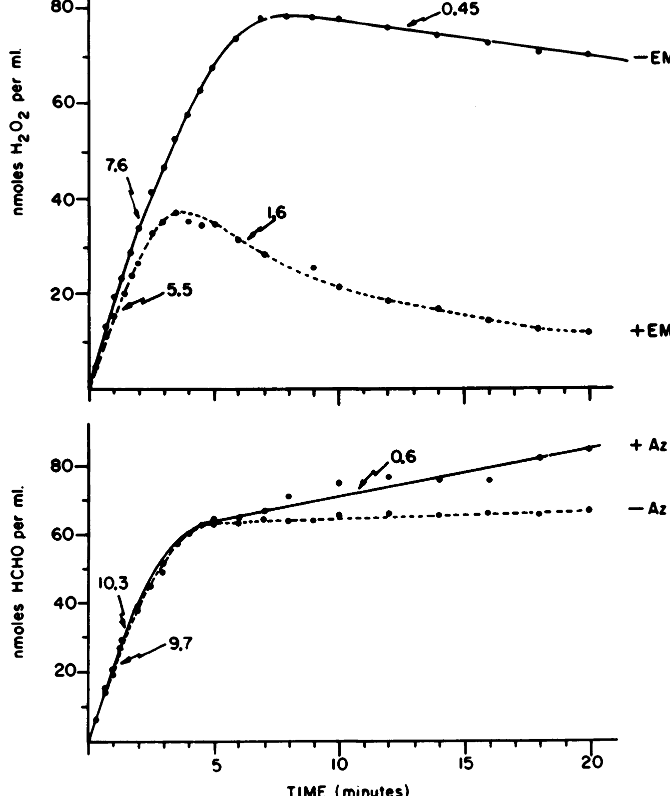

Figure 10. The influence of ethylmorphine on the rate and extent of hydrogen peroxide formation in the presence of a limiting concentration of NADPH.

A series of experiments similar to those described in Figure 5 were carried out in the presence or absence of 5mM ethylmorphine or 1mM sodium azide. The amount of hydrogen peroxide or formaldehyde formed were determined as described in Figure 6. An NADPH generating system was omitted during this series of experiments. The reactions were initiated by addition of 145μM NADPH.

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compounds that result in an increased rate of formation of hydrogen peroxide where the increased rate of utilization of oxygen is accompanied by an equivalent increase in the rate of NADPH oxidation (such as hexobarbital or benzphetamine) and a second type of "uncoupling" where non-metabolized substrates such as perfluorinated, aliphatic fluorocarbons (45) or compounds like halothane (Werringloer, J and Estabrook, R.W., unpublished results) result in a failure to see any additional hydrogen peroxide formed upon stimulation of oxygen utilization, i.e., two moles of NADPH are utilized for each additional mole of oxygen reduced offsetting the stoichiometry of 1 mole of oxygen utilized per mole of NADPH oxidized. The details of how "uncouplers" divert the function of cytochrome P-450 in a presumed abortive reaction resulting in the dissipation of the oxycytochrome P-450 ternary complex remains as a challenge for further experimental examination.

Electron paramagnetic resonance studies. The above discussion centers on the potential role of a superoxide anion as an intermediate in the formation of hydrogen peroxide during NADPH oxidation by liver microsomes. Since the superoxide anion is a free radical it should be detectable by electron paramagnetic resonance spectroscopy (77). When liver microsomes are incubated in an oxygen saturated buffer in the presence of sodium azide and a sample is rapidly frozen in liquid nitrogen soon after initiation of the reaction by the addition of NADPH, examination by EPR spectroscopy reveals the formation of a signal at about g = 2.0 as shown in Figure 12. The generation of this new EPR signal, however, does not appear to be related to hydrogen peroxide formation or the generation of a free radical of the superoxide anion type. The same EPR signal at g = 2.0 is obtained if NADH is employed rather than NADPH and the signal is unaltered if sodium azide is omitted from the medium when reduced pyridine nucleotide is added. Further the signal remains when the frozen sample of microsomes is warmed from the temperature of liquid nitrogen to -10° and its power saturation characteristics resemble those described for a similar free radical signal described by Iyanagi and Mason (78) which they attributed to a flavin free radical generated during the function of the microsomal flavoprotein, NADPH-cytochrome c reductase. Thus no positive evidence for the presence of the superoxide anion could be obtained by examining liver microsomes by EPR spectroscopy. A number of explanations could be offered to rationalize this negative result, such as the presence of an active superoxide dismutase associated with the microsomal fraction; evenso, this approach to define the presence of the superoxide anion does remain to be further explored.

NADH Synergism. A number of years ago (47,48) it was recognized that NADH oxidation by liver microsomes concomitant

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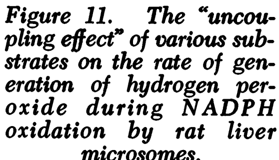

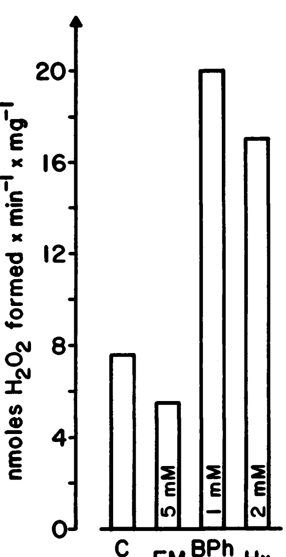

Figure 11. The "uncoupling effect" of various substrates on the rate of generation of hydrogen peroxide during NADPH oxidation by rat liver microsomes.

A series of experiments were carried out using liver microsomes from phenobarbital-treated rats incubated in a reaction mixture as described in Figure 7 containing an NADPH generating system. Where indicated 5mM ethylmorphine (EM), 1mM benzphetamine (BPh), or 2mM hexobarbital (Hx) were added to the reaction mixture.

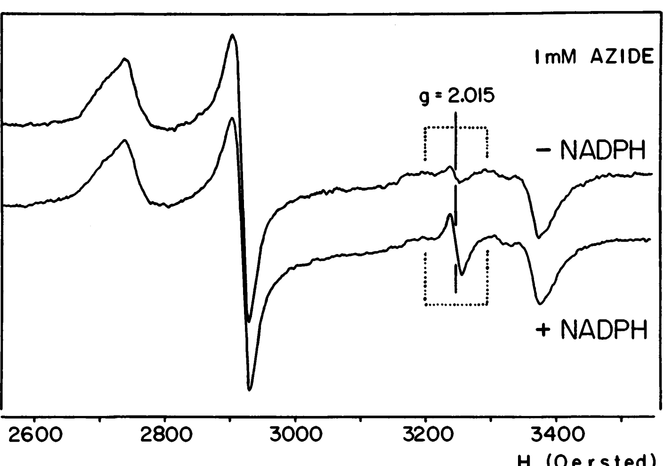

Figure 12. Changes in the electron paramagnetic resonance signals of liver microsomes associated with the initiation of NADPH oxidation by liver microsomes.

Liver microsomes from phenobarbital-treated rats were suspended at 10 mg protein per ml in an oxygenated reaction medium containing 50mM tris-chloride buffer, pH 7.5, 150mM KCl, 10mM $MgCl_2$, and 1mM sodium azide. An aliquot was removed, placed in a calibrated EPR tube, and rapidly frozen in liquid nitrogen (upper curve). NADPH (final concentration, 200μM) was then added to the suspension and an aliquot removed and frozen within 15 sec (lower curve). First derivative spectra were obtained with an E-4 Varian EPR with the samples maintained at the temperature of liquid nitrogen.

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with the oxidation of limiting concentrations of NADPH resulted in a marked enhancement in the oxidative metabolism of substrates of cytochrome P-450 such as aminopyrine and ethylmorphine. As shown in Figure 13 the initial rate of N-demethylation of ethylmorphine by rat liver microsomes is nearly doubled when NADH is added in the presence of NADPH. This stimulation of activity is reflected not only in the rate but also the extent of product formed. This observed effect of NADH greatly exceeds an additive effect of the action of the two forms of reduced pyridine nucleotides since the N-demethylation of ethylmorphine is relatively slow in the presence of NADH alone. This type of experiment together with measurements on changes in the extent of steady state reduction of cytochrome $\underline{b_5}$ (51) have served as one of the foundations for the development of the cyclic scheme of cytochrome P-450 function as described in Figure 4. It is proposed that NADH serves to donate, via the flavoprotein reductase and cytochrome $\underline{b_5}$, the electron required to reduce oxycytochrome P-450 to an intermediate with an oxidation state equivalent to a peroxide anion form.

Of interest was the question whether a similar NADH synergism would be observed during the generation of hydrogen peroxide associated with the oxidation of NADPH. In this way it may be possible to gain some insight into which form of the oxygen complex of cytochrome P-450 serves as the source of hydrogen peroxide.

As shown in Figure 14, NADH does not have a significant synergistic affect on the generation of hydrogen peroxide during NADPH oxidation. A small increase in the initial rate of hydrogen peroxide formation is observed in the presence of NADH and NADPH, relative to the rate observed when NADPH alone is used as the donor of reducing equivalents, but this increase is approximately equal to the rate observed when NADH is used to support the reaction. The extent of hydrogen peroxide formation observed does increase measurably but this may be attributed in large part to a "sparing effect" of reducing equivalents from NADPH used to support the mixed function oxidation of "endogenous substrates" as described in an earlier section of this paper. On the basis of these results it is concluded that the same type of NADH synergistic effect characteristic of hydroxylation reactions mediated by cytochrome P-450 does not function for the generation of hydrogen peroxide. This observation may result from an alteration of the role for a needed second electron to form the peroxide anion form of cytochrome P-450 or from the dominant role of the dissociation of oxycytochrome P-450 giving rise to the superoxide anion. The latter hypothesis would preclude the need for donation of a second electron in the formation of hydrogen peroxide.

The peroxidatic function of cytochrome P-450. Hrycay and O'Brien (54-56) have described experiments which demonstrate the

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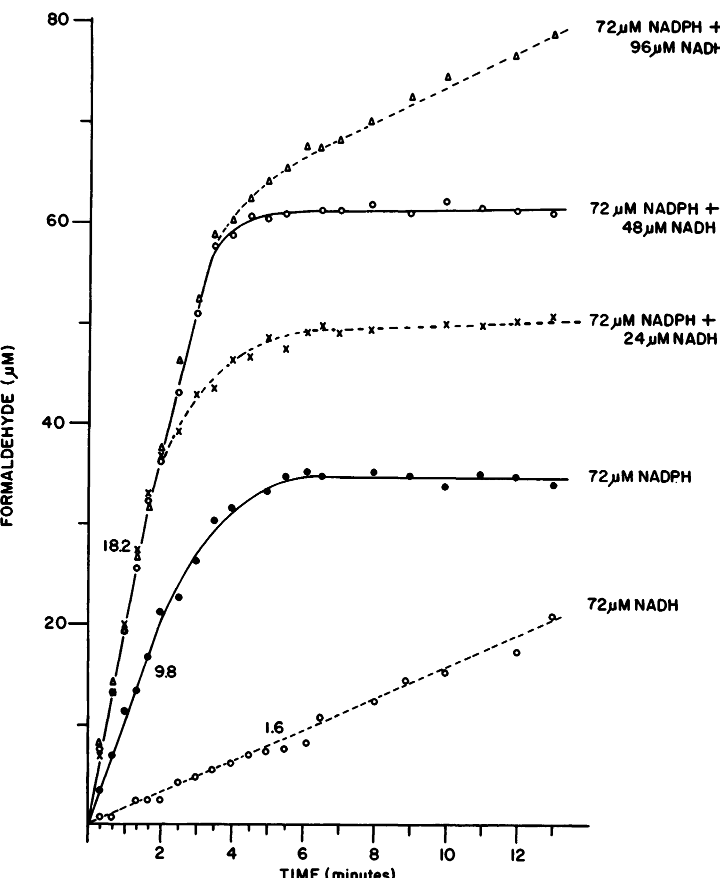

Figure 13. The synergistic effect of NADH on the NADPH-dependent N-demethylation of ethylmorphine.

Liver microsomes from phenobarbital-treated rats were incubated at 1 mg of protein per ml in the presence of 5mM ethylmorphine. NADPH and NADH were added in the concentrations indicated to initiate the reaction. Samples were removed and the amount of formaldehyde formed determined by the Nash reagent (84). Sodium azide and an NADPH generating system were omitted from the reaction medium.

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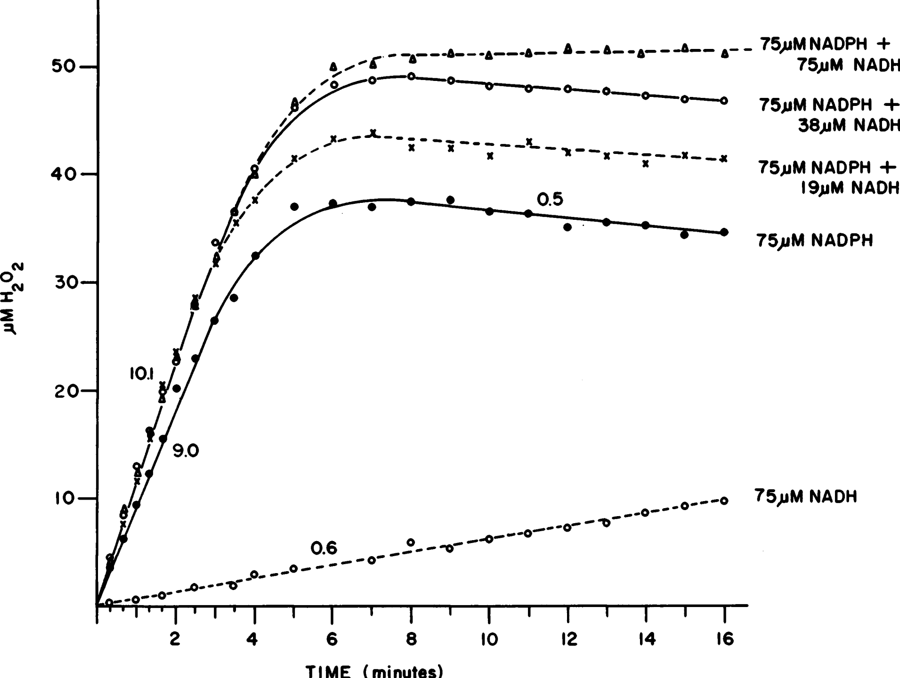

Figure 14. The effect of varying concentrations of NADH on the rate and extent of formation of hydrogen peroxide associated with the oxidation of a limiting concentration of NADPH by liver microsomes. A series of experiments similar to those described in Figure 5 were carried out in the presence of NADPH and NADH as indicated.

ability of cytochrome P-450 of liver microsomes to serve as a peroxidase. About the same time, Kadlubar et al (57) demonstrated the ability of organic hydroperoxides to support N-demethylation reactions when liver microsomes are added to the reaction mixture. Recently considerable interest has centered on the mechanism of these peroxide catalyzed reactions as they involve cytochrome P-450 since such reactions may provide a different means of evaluating the presence and nature of "active oxygen".

When liver microsomes interact with ethylmorphine and hydrogen peroxide, in the presence of sodium azide to inhibit contaminating catalase, one observes (Figure 15) a stoichiometric utilization of hydrogen peroxide concomitant with the formation of formaldehyde - the product of N-demethylation of ethylmorphine (58). This reaction is not dependent on the presence of oxygen and is not inhibited by carbon monoxide. Relatively high levels of hydrogen peroxide are required to obtain maximal rates of the N-demethylation reaction and an apparent Km of approximately 20 mM for hydrogen peroxide has been determined (58). Under optimal conditions rates of N-demethylation are observed in the presence of hydrogen peroxide which are greater than 50 fold the rate obtained when NADPH supports the oxidative transformation of ethylmorphine.

Both optical (Figure 16) and electron paramagnetic resonance (Figure 17) spectroscopy studies (59) revealed changes in the oxidation properties of microsomal cytochrome P-450 upon addition of organic hydroperoxides. Transient changes in the optical spectra were observed upon addition of cumene hydroperoxide and these spectral changes differed from those reported (44) for oxycytochrome P-450 observed during the aerobic steady state oxidation of NADPH by liver microsomes. Further, electron paramagnetic resonance studies (Figure 17) revealed the formation of EPR signals in the area of $g = 2.0$ different from those described in Figure 12. Indeed, the unique nature of the EPR signals observed at about $g = 2.0$ when cumene hydroperoxide reacts with microsomal cytochrome P-450 resemble the signals observed when hydrogen peroxide reacts with metmyoglobin or cytochrome c peroxidase (79, 80). From these results it was concluded (59) that cytochrome P-450 may obtain higher valence states of the heme iron in a manner analogous to that proposed by Yamazaki et al (81) for peroxidases. This would suggest that the equivalent of an "oxene" form of oxygen might be formed during the activation of oxygen by cytochrome P-450 as it functions in hydroxylation reactions. It is of interest to note that the EPR signal observed when peroxides interact with liver microsomal cytochrome P-450 is very similar to that reported by Vanneste et al (82) for the complex of oxygen with reduced cytochrome P-450 obtained in rapid freeze quenching experiments.

Concluding Remarks. The experimental results described in the preceeding sections have been presented to provide some background as to the present status of our understanding of "active

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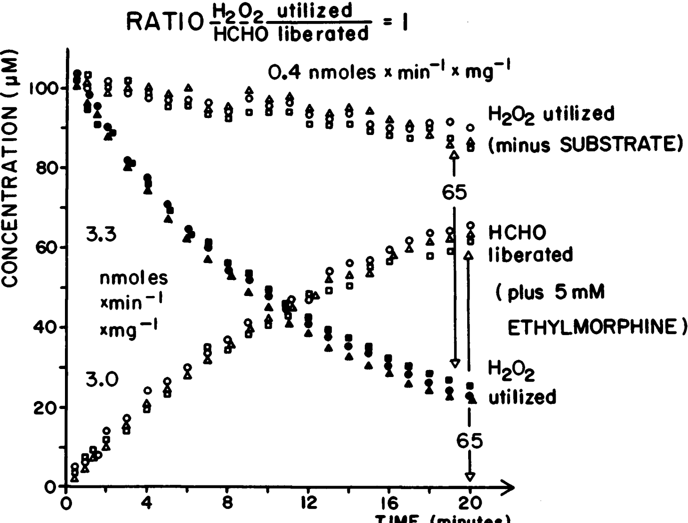

Figure 15. The hydrogen peroxide-dependent N-demethylation of ethylmorphine as catalyzed by liver microsomes.

Liver microsomes from phenobarbital-treated rats were diluted to 2 mg protein per ml in a reaction mixture similar to that described in Figure 5. Where indicated, 5mM ethylmorphine was added. The reaction was initiated by adding 100μM hydrogen peroxide. Special reaction vessels were used to permit equilibration with various gas mixtures and to permit sampling the reaction at the times indicated. The changes in the concentrations of hydrogen peroxide used or formaldehyde formed were determined colorometrically.

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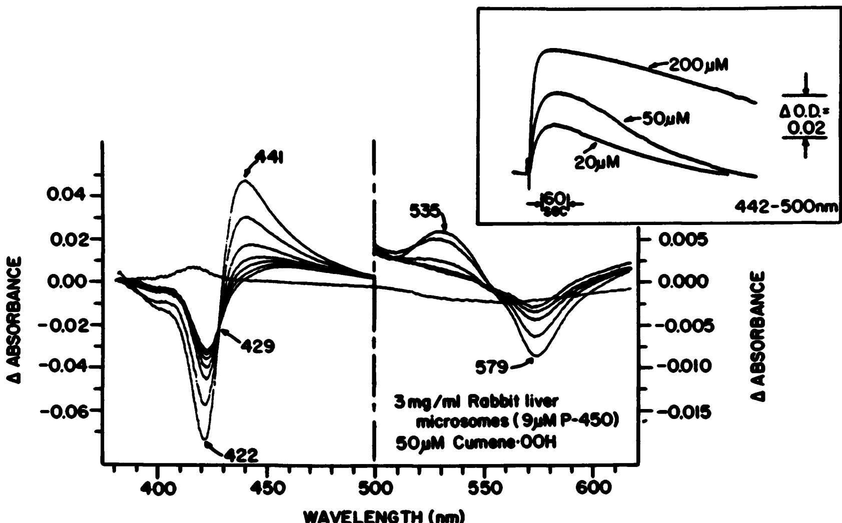

Figure 16. Spectrophotometric measurement of changes occurring during the oxidation of cumene hydroperoxide by rat liver microsomes.

Liver microsomes from phenobarbital-treated animals were diluted to a protein concentration of 3 mg per ml in a reaction mixture containing 50mM tris-chloride buffer, pH 7.5, 150mM KCl, and 5mM $MgCl_2$. After recording a baseline of equal light absorbance, 50μM cumene hydroperoxide was added to the contents of the sample cuvette, and the change of absorbance with time was determined by repetitive scanning at 2 nm per sec. (Insert) The kinetics of formation and decay of the absorbance at 442 nm at different concentrations of cumene hydroperoxide.

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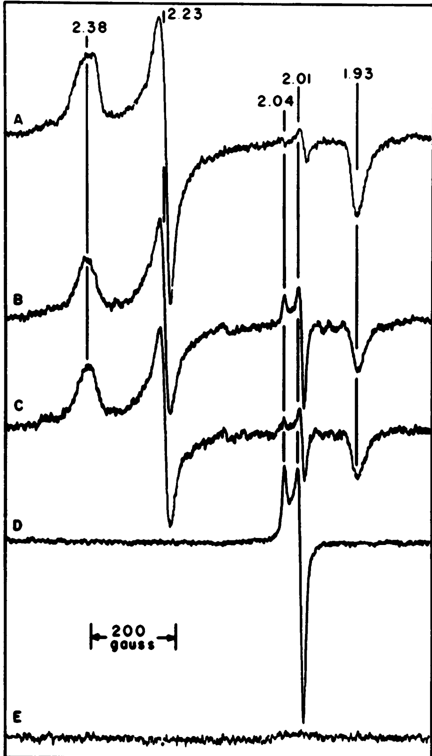

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Figure 17. Changes in the EPR spectra associated with the interaction of cumene hydroperoxide and liver microsomes.

Experiments were carried out using liver microsomes from phenobarbital-treated rabbits. (A) no additions; (B) after adding 1.5mM cumene hydroperoxide; (C) after adding 46mM cyclohexane followed by cumene hydroperoxide; (D) 50μM metmyoglobin mixed with 1.5mM cumene hydroperoxide; (E) a baseline obtained in the absence of liver microsomes (59).

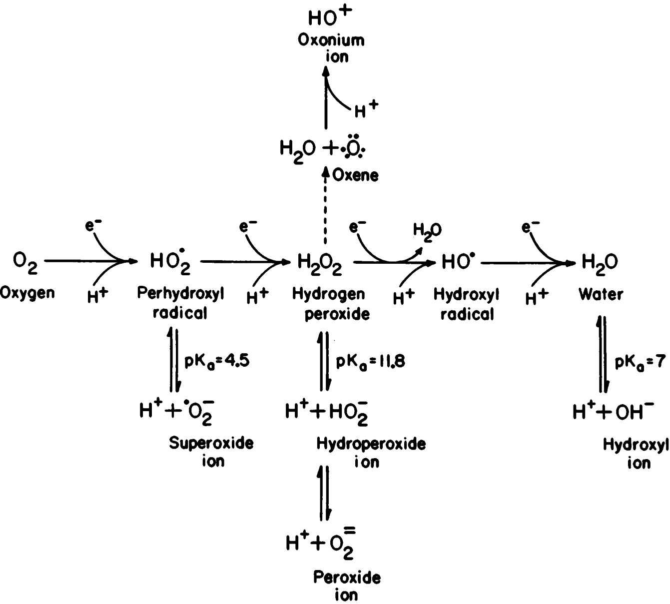

Figure 18. The possible various states of oxygen during its stepwise reduction to water

oxygen" formed during cytochrome P-450 catalyzed reactions. Oxygen may exist in a number of oxidation states as illustrated in Figure 18. As yet it is not possible to assign a specific function in hydroxylation reactions for the superoxide anion, peroxide anion, oxene or their protonated forms. The ability to observe the formation of hydrogen peroxide during NADPH oxidation by liver microsomes and to assign a role for cytochrome P-450 in the generation of hydrogen peroxide lends credence to the scheme proposed in Figure 4. Further the experimental observations obtained during the peroxidatic function of cytochrome P-450 all point to the central role for a peroxide anion complex of this pigment playing a central and pivitol role in the activation of oxygen. New approaches and more experiments will be required to better establish the validity of the current hypotheses on the proposed intermediates formed during cytochrome P-450 function.

Oxygen is central to the maintainances of the life of higher organisms as we now understand it. In addition to reactions in the cell where oxygen is reduced to water concomitant with the conservation of energy in the form of ATP, as catalyzed by the mitochondrial respiratory chain, oxygen plays a key role in the synthesis and degradation of a wide diversity of natural compounds, such as steroids, as well as foreign chemical agents. For these latter reactions cytochrome P-450 plays a critical role serving to activate oxygen for interaction with these organic substrates. Undoubtably the future will provide many new surprises as we delve deeper to gain a fuller understanding of this important enzyme system.

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2

Synthetic Models for the Reaction Stages of Cytochrome P-450

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Metalloenzymes often exhibit primary coordination spheres which have no counterparts among structurally-characterized, synthetic complexes. The physical and chemical properties of these metal centers may also be unusual and may not have analogies to the corresponding properties of synthetic coordination compounds. The relationship between protein structure and these uncommon physical and chemical properties is essential to understanding the functions of metal protein active sites. In this paper I will summarize the work of our group in characterizing model complexes for the iron center in three of the five recognized reaction stages of the hemoprotein cytochrome P-450 family (1, 2). This work is far from complete so that the present paper is a progress report. Many other laboratories have also contributed to modelling these P-450 stages, but the present account will not attempt to review the contributions of other groups in any depth.

Consider a simplified reaction cycle for the soluble, bacterial camphor hydroxylase, P-450$_{cam}$, from Pseudomonas putida (3). Using the highly purified soluble P-450$_{cam}$ system, Gunsalus, et al. have been able to reassemble components of the enzyme system in vitro and thus to observe and to characterize four stable intermediates in the P-450$_{cam}$ cycle as well as the inactive ferrous carbonyl stage (4). These studies have resulted in the elucidation of the reaction sequence shown in Figure 1. It should be emphasized that the bacterial P-450$_{cam}$ cycle shown in Figure 1 differs in some degree from that of the membrane bound microsomal cycle deduced by Coon (5, 6). The electron transport components which are different in the P-450$_{cam}$ and P-450$_{lm}$ systems are omitted here as these are far beyond the present scope

of modelling. Because of their prior recognition, the stages in the P-450$_{cam}$ cycle (Figure 1) served as targets for the modelling studies to be described herein. Figure 2 shows a simplified version of the reaction cycle along with the distinctive properties associated with each stage which are necessary to provide for assessing the value of any synthetic model. It is clear that there are strong similarities between recognized reaction stages of the P-450$_{cam}$ and the P-450$_{lm}$ reaction cycles.

Before discussing models for these stages of cytochrome P-450, it is appropriate to review the structural characteristics of low and high spin iron porphyrins. These relationships which were first predicted by Hoard (7) are now well established and have no exceptions among structurally characterized synthetic porphyrins. The situation is summarized in Table I. Ferrous iron has six d electrons. In the

Table I

Structural Characteristics of Iron in Hemoproteins

Biological Example	Oxidation and Spin State			Coord. No.	Expected Fe-N Å
Mb, Hb, P^{C}_{450}	Fe^{2+}	d^6	S=2	5	2.09
MbO_2, HbO_2, HbCO $P_{450}O_2$, $P_{450}CO$	Fe^{2+} Fe^{3+}	d^6	S=0	6	2.00
P^{B}_{450} (substrate)	Fe^{3+}	d^5	S=5/2	5	2.07
P^{A}_{450} (resting)	Fe^{3+}	d^5	S=1/2	6	1.99

Radius of porphyrin core ~2.01.

diamagnetic (S=0) state, iron(II) porphyrins possess two axial ligands affording overall six-coordination. Low spin iron(II) has a covalent radius which fits without stress into the porphyrin core. Well-established examples are diamagnetic ferrous carbonyl derivatives similar to stage D in Figure 1. High spin iron(II) has four unpaired electrons (S=2) and a covalent radius too large to be accommodated by the porphyrin core. Such high spin ferrous complexes have a single axial ligand (overall 5-coordinate) and iron is displaced out of the plane of the porphyrin ring as, for example, in deoxymyoglobin. The ferric state

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with five d electrons is structurally similar to the ferrous state. Low spin ferric porphyrins have one unpaired electron ($S=\frac{1}{2}$), two axial ligands, and in-plane iron. High spin ferric porphyrins have five unpaired electrons, ($S=5/2$), a single axial ligand, and iron out of the porphyrin plane.

Low Spin Ferric Stage A. Consider the resting stage of cytochrome $P\text{-}450_{cam}$, stage A, Figure 2. The heme iron is in the low-spin ferric state and must, therefore, have two axial ligands. Mason was the first to recognize that the axial ligation in P-450 is unconventional and to suggest sulfur coordination (8,9). The esr spectra of this low spin form have unusual rhombic g values which are similar to the g values afforded by addition of thiols to methemoglobin and myoglobin (10,11).

On the basis of model complexes generated *in situ* by our group (12) and that of my colleague, Holm (13), it seems certain that stage A has one axial thiolate ligand and another unspecified axial ligand. The thiolate ligand undoubtedly results from the mercaptide anion of cysteine. Solutions of thiolate complexes of ferric porphyrins are intrinsically unstable, spontaneously affording disulfide and ferrous porphyrins as shown in eq. 1. The scope and

$$2RS\text{-}Fe^{III}(P)(B) + 2B \longrightarrow R\text{-}S\text{-}S\text{-}R + 2\ BFe^{II}(P)(B) \quad (1)$$

(P represents any porphyrinato group and B any axial base).

detailed mechanism of this redox reaction is at present unclear. The protein and/or lipid membrane of cytochrome P-450 must somehow serve to inhibit this redox process. Synthetic models offer a clue as to the inhibition of this reaction. We have been able to prepare a stable, crystalline ferric thiolate complex, **1**, as illustrated in eq. 2. The

$$[Fe^{III}TPP]_2O \xrightarrow[H_2SO_4,\ C_6H_6]{C_6H_5SH} (C_6H_5S)Fe(TPP)(HSC_6H_5) \quad (2)$$

1

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complex Fe(TPP)(SC_6H_5)(HSC_6H_5) is very unusual in that it is the only isolated low-spin thiolate ferric porphyrin and the only isolated ferric porphyrin having two different axial ligands to our knowledge. In solution this thiolate complex is unstable and undergoes a redox disproportionation reaction similar to that shown in eq. 1. However in the crystalline state, the ferric thiolate complex **1** is quite stable. The physical properties of **1** are very unusual and offer a possible analogy to the facile spin equilibrium exhibited by cytochrome P-450 systems which pass from low to high spin ferric (stage A to B) upon substrate binding. Single crystals of **1** are predominately high spin ($\mu = 5.4$ BM) at 25°; however, as the temperature is lowered, a low spin form is observed which eventually becomes dominant. This unusual temperature-dependent equilibrium is quite reversible and does not affect the mosaicity of single crystals. We have been monitoring this change with a battery of physical techniques which include X-ray diffraction, Mössbauer spectroscopy, esr, and magnetic measurements (14). These studies are still in progress but some points are becoming clear. The esr of the dominant high spin form has g values (~8.6, ~3.4) comparable with those of the substrate-bonded P-450 (stage B). At -196° the esr shows a dominant low spin form whose g values (2.40, 2.25, 1.97) are similar to those of the resting form of P-450 (stage A). X-ray diffraction at -160° shows the low spin form to represent ~70% of the molecules. This result is consistent with preliminary Mössbauer studies which indicate that a spin equilibrium is occurring between two spin states of a single species. Two very different iron-sulfur distances are apparent from the X-ray data at 25°, consistent with the assumption that one sulfur is not coordinated in the high spin form.

Under vacuum **1** loses benzenethiol, producing the high spin complex **2**, which has a single axial thiolate ligand (eq. 3). Small gaseous ligands such as ammonia

S
Fe
S
H
1
vacuum
100°
S
Fe
2
(3)

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and methylamine will penetrate solid samples of 2 affording low-spin complexes with esr g values similar to those of stage A of the P-450 cycle (Figure 3). Solutions of 2 in the presence of axial bases are unstable with respect to the redox reaction (eq. 1). However, when cold toluene solutions of 2 are mixed with various axial bases and then frozen, the resulting glasses exhibit esr g values typical of the low spin stage A of P-450. Similar studies have been carried out by Holm (13) who employed protoporphyrinIX dimethyl ester, PPIXDME, rather than tetraphenyl porphyrin, TPP. Table II exhibits representative g values for low-spin ferric porphyrins having different combinations of axial ligation along with representative values for cytochrome P-450 and cytochrome c. Comparison of these values clearly indicates that one axial thiolate ligand in combination with virtually all other possible modes of ligation will afford g values similar to those found for the resting stage A of P-450. However the g values for other combinations of ligation without an axial mercaptide do not correspond as closely to those of P-450. On the basis of such *in situ* studies we conclude that one axial ligand in stage A of P-450 is very probably the cysteine thiolate but the other axial base cannot be distinguished from the following possibilities: oxygen (water, amide carbonyl, serine hydroxyl), nitrogen (imidazole or lysine amino), or neutral sulfur (methionine thioether or cysteine thiol). The role of sulfur as one ligand in stage A is also consistent with Peisach's recent studies using esr in an electric field (15) and with Holm's limited study of low temperature UV-visible spectra of model compounds generated *in situ* (13).

One experiment provides a possible explanation for the stabilization of the low spin ferric-thiolate porphyrin by the protein or the lipid in cytochrome P-450, stage A. Reaction between cold toluene solutions of the high-spin complex 2 and polystyrene bonded imidazole affords a solid low-spin polymeric complex, $FeTPP(SC_6H_5)$(N N-polystyrene), 3, which is stable for months at 25°C in the absence of solvent (12). It is apparent that immobilization kinetically stabilizes such low spin thiolate complexes.

In the same vein, a mercaptopropyl group covalently attached to silica gel reacts with $[FeP]_2O$ in the presence of a base B (B=pyridine or N-MeIm) to give stable species with epr g values identical to those of the corresponding complexes generated *in situ*

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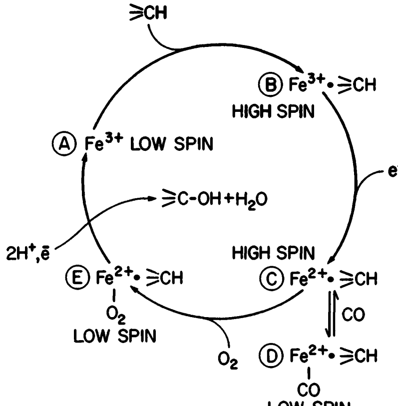

Figure 1. The reaction cycle for P-450_{cam}. ⩾CH is the substrate molecule, camphor.

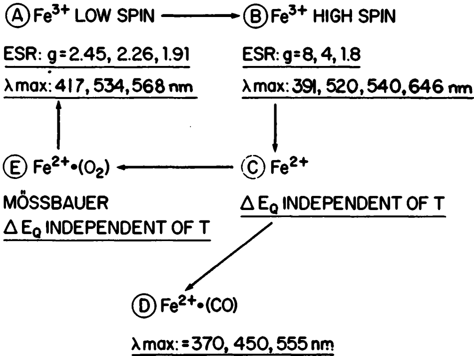

Figure 2. Distinctive properties for the reaction stages of P-450_{cam}

S III Fe —B, TOLUENE, −50°→ S III Fe B

B SOLID 25°

B = N-methylimidazole, tetrahydrofuran, tetrahydrothiophene, camphor, Ⓟ-imidazole

S III Fe B

B = NH_3, CH_3NH_2

Figure 3. Scheme for the preparation of models for the resting stage Ⓐ of P-450

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Table II
EPR Spectra for Low Spin Ferric Porphyrin Complexes, Fe(P)(X)(B)[a,b]

X	B	Donor Set	g_1	g_2	g_3
SC_6H_5	N-MeIm	S^-, N	2.39	2.26	1.93
SC_3H_7	N-MeIm	S^-, N	2.37	2.25	1.95
$OC_6H_4NO_2$	N-MeIm	O^-, N	2.61	2.21	1.85
OCH_3	N-MeIm	O^-, N	2.43	2.15	1.92
N-MeIm	N-MeIm	N, N	2.90	2.29	1.57
SC_6H_5	CH_3NH_2	S^-, N	2.38	2.22	1.96
SC_6H_5	THF	S^-, O	2.34	2.25	1.96
SC_6H_5	camphor	S^-, O	2.37	2.27	1.94
SC_6H_5	HSC_6H_5	S^-, S	2.40	2.25	1.97
SC_6H_5	THT	S^-, S	2.36	2.27	1.95
	P-450 (cam)	S^-, ?	2.45	2.26	1.91
	P-450 (TBM)	S^-, ?	2.40	2.25	1.93
	cytochrome C	S, N	3.06	2.25	1.24

(a) Most spectra were obtained in frozen toluene glasses at -196°.

(b) P=TPP; other porphyrins give similar results (ref. 13).

at low temperature (Figure 4)(16). Attempts to remove the base B under vacuum have been marginally successful, leading to some decomposition of the porphyrin. Further work aimed at preparing the five-coordinate alkyl thiolate complexes immobilized on silica is still in progress, as well as parallel studies on thio methylpolystyrene, Ⓟ—CH_2SH (16).

High Spin Ferric Stage B. Upon substrate binding, the resting low-spin form A of P-450_{cam} is converted into a high-spin ferric stage B (Figure 1). The unusual epr and electronic spectral characteristics of B have been reproduced in model ferric porphyrins having a single axial thiolate ligand (12,13,17). Examples of such isolated ferric complexes are presented in Table III. It is interesting to note that Fe(OEP) and Fe(PPIXDME) form five-coordinate thiolate complexes directly whereas with Fe(TPP), the six coordinate species 1 is obtained which must be further treated to give the desired high-spin adduct. The dissimilar physical properties (Table III) exhibited by these complexes may reflect some crystal packing forces which are a function of the mode of preparation.

Holm, et al. have characterized by X-ray diffraction one such model complex, Fe(PPIXDME)-($SC_6H_4NO_2$), 4, and studied this compound with a wide range of spectroscopic techniques (13). He found that distinctive features in the uv-visible, MCD, epr, and Mössbauer spectra of 4 closely parallel the analogous spectral features of cytochrome P-450_{cam} stage B whereas other axial ligands gave less satisfactory correspondence. Our own more limited studies (12) show that Fe(TPP)(SC_6H_5), 2, has epr g-values similar to those of stage B, although our complex is less well defined due to its relative instability. In fact, even at low temperature, Fe(TPP)(SC_6H_5) undergoes a slow decomposition to give a species with epr g-values of 6.0 and 2.0. Thus, the absorbances of $g=8.6$ and $g=3.4$ appear as shoulders on the $g=6$ peak and can only be approximated; in reality, they are probably closer to 8 and 4. Another consequence of the instability of 2 is the appearance of a broad absorption at $g = 2$ which obscures the high field region and the expected third resonance at $g=1.8$. Table IV shows that no other type of experimentally accessible axial ligation will produce similar epr spectral features.

Ogoshi has prepared a model for stage B in which the axial ligand is an alkyl thiolate (17) (Table III)

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Table III
Models for Substrate Bonded High Spin Ferric P-450

Complex	μ (B.M.)	g Values	Ref.
$FeTPP(SC_6H_5)$	5.8	~8.6, ~3.4, --[a]	12
$FePPIXDME(SC_6H_4Cl)$	5.9	7.2, 4.8, 1.8	13
$Fe(OEP)(SC_6H_5)$	5.9	7.2, 4.7, 1.9	13
Fe(OEP)(t-BuS)	5.9	6.4, 4.4, 2.0	17
$P\text{-}450_{cam}$·substrate	5.2	8.0, 4.0, 1.8	3

(a) See text for explanation.

Table IV
EPR Data for High Spin Ferric Porphyrin Complexes

Complex	Donor	g_1	g_2	g_3	Ref.
$FeTPP(SC_6H_5)$	S^-	~8.6	~3.4	--	12
$FePPIXDME(SC_6H_4Cl)$	S^-	7.2	4.8	1.8	13
FeOEP(S-t-Bu)	S^-	6.4	4.4	2.0	17
$FeTPP(OCH_3)$	O^-	6.6	5.3	1.9	16
$FePPIXDME(OC_6H_4NO_2)$	O^-	5.9	5.9	2.0	13
$FePPIXDME(O_2CCH_3)$	O^-	5.9	5.9	2.0	13
Met-Hb	N	6.0	6.0	2.0	18
P-450 (TBM)	S^-	8.3	3.3	--	19
P-450 (cam)	S^-	8.0	4.0	1.8	3

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in contrast with the aryl thiolates employed by Holm, *et al.* and our own group. Such alkyl thiolate ferric porphyrins are far less stable than the aryl analogues with regard to the self-degradative redox reaction such as that shown in eq. 1, which led us to our attempts to immobilize such complexes on silica. The epr *g* parameters reported by Ogoshi are not as similar to those of stage B as those of the aryl thiolate complexes; however, Ogoshi's *g* values are somewhat suspect due to the unusual splitting of the low field resonance. In view of this discrepancy it seems desirable to examine additional models for stage B using alkyl thiolates.

Stage C. To date, well defined five-coordinate high-spin ferrous porphyrins having an axial thiolate or an axial thiol ligand have not been isolated or even characterized *in situ* so that comparison cannot be made with the unique physical properties of the iron center in cytochrome $P\text{-}450_{cam}$ stage C (Figure 2). Recent Mössbauer studies of stage C in the presence of strong applied magnetic fields have shown distinctive parameters which have so far not been reproduced by model studies and are different from those properties of deoxymyoglobin in which imidazole is the axial ligand (20).

Stage D. The ferrous carbonyl form (stage D) of cytochrome P-450 is characterized by an unusual Soret at about 450 nm which is red shifted from the Soret of "normal" ferrous carbonyl hemes which appears around 420 nm. The unusual 450 nm absorption has been invaluable as an analytical aid for determining the presence of this cytochrome. Small differences in the position of this band (from 446 to 453 nm) have been observed in the spectra of different members of the cytochrome P-450 family. Hanson, *et al.* (21) have proposed a theoretical explanation for this unusual Soret on the basis of theoretical calculations and the experimental observation of another optical transition at 363 nm having the same polarization as the 446 peak in the bacterial cytochrome $P\text{-}450_{cam}$. These "hyperbands" can be explained by an interaction between a $p \longrightarrow e_g\ (\pi^*)$ (porphyrin) and the normal porphyrin $a_{1u}(\pi)$, $a_{2u}(\pi) \longrightarrow e_g\ (\pi^*)$ Soret transition resulting in the observed pair of bands. Such transitions could be caused by binding of an axial thiolate ligand.

Stern and Peisach first reported that the unusual 450 nm Soret could be generated *in situ* in an

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Fe(PPIX) thiol/CO DMSO-EtOH solution under very strongly basic conditions (22). Interpretation of their data is complicated by the large excess of thiol and base required, the potential for coordination of the DMSO and ethanol solvent, and the presence of another "normal" Soret, as well as temporal characteristics of the spectrum. Under conditions of controlled stoichiometry and solvent environment we have been able to quantitatively reproduce the full electronic and magnetic circular dichroism spectra of stage D in the P-450_{cam} sequence. This was accomplished by combining equivalent amounts of the sodium crown-ether methyl mercaptide salt with iron(II) porphyrins and CO in anhydrous benzene as shown in Figure 5 (23). This ferrous carbonyl complex is extremely sensitive towards oxygen. The infrared νCO band at 1945 cm^{-1} is close to the 1938 cm^{-1} value reported by Caughey, *et al*. for the substrate bonded stage D, P-450_{cam} (24). However this similarity may not be meaningful in that several factors such as local polarity and tilting of the CO group from the axis normal to the porphyrin can influence νCO values (25).

The electronic and MCD spectra (26) of our mercaptide ferrous carbonyl porphyrins generated *in situ* are displayed along with those of highly purified P-450_{lm} in Figures 6 and 7. The quantitative similarity between the PPIXDEE derivative and the natural system is striking. The spectral band at 370 nm, first described and explained by Hanson, *et al*. (21) is clearly evident in each. These spectral comparisons provide strong evidence that an axial mercaptide ligand is present in stage D of cytochrome P-450. It is noteworthy that no other ligand (RO^-, PhO^-, RCO_2^-, RSH, imidazole, $RCONH_2$, ROH, or RNH_2) is capable of producing this unusual chromophore. We also discovered that the 449 nm Soret could be shifted to longer wavelengths by increasing the polarity of the solvent. This observation provides a possible explanation for the variation in the Soret of stage D observed for different members of the P-450 family. Subsequently, Dolphin, *et al*., reported similar optical spectra for models of stage D generated *in situ* (27). We are currently attempting to prepare a crystalline model for stage D.

Various reagents denature cytochrome P-450 yielding a protein whose ferrous carbonyl exhibits a Soret band at 420 nm. We have reproduced the MCD spectrum of this so-called P-420 by combining Fe(II)PPIXDEE, CO, and *either* N-methyl imidazole *or* an alkyl mercaptan (26). These spectra are shown in

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$(SiO_2)_X + (EtO)_3{-}SiCH_2CH_2CH_2SH \xrightarrow{-3\,EtOH}$

$(SiO_2)_X - SiCH_2CH_2CH_2SH \xrightarrow[B]{(Fe\,PPIXDME)_2O}$

$(SiO_2)_X - SiCH_2CH_2CH_2S{-}Fe{-}B$

EPR: B = PYRIDINE, g = 2.34, 2.25, 1.96
B = N-MeIm, g = 2.38, 2.26, 1.95

Figure 4. Scheme for immobilization of low spin ferric porphyrin thiolate complexes on silica gel [$(SiO_2)_X$]

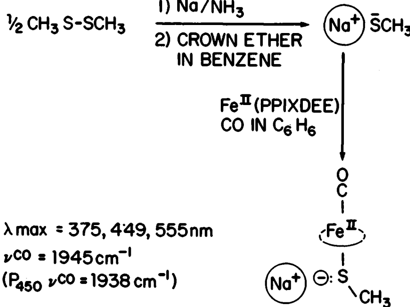

Figure 5. Scheme for the preparation of a model for the carbonyl stage Ⓓ of P-450

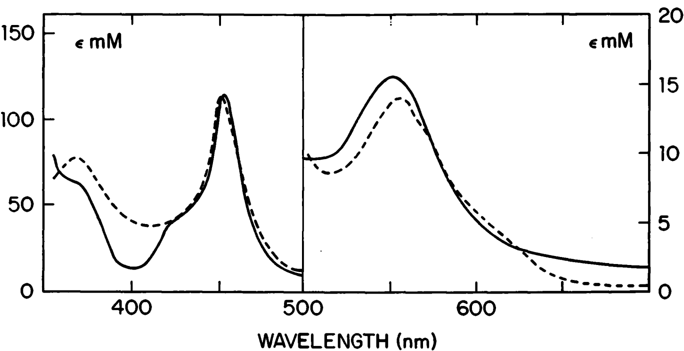

Figure 6. Absorption spectra for highly purified P-450$_{LM}$ carbonyl (——) and FePPIXDEE + Ⓝⓐ SCH_3 + CO in benzene (- - -)

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Figure 8. This result indicates that the nature of the axial ligand in P-420 cannot be assigned at present and raises the possibility that P-420 could represent a variety of different modes of axial ligation. However these results demonstrate that stage D of P-450 does not have an axial thiol ligand.

Oxygenated Stage E. The nature of the other axial ligand in the cytochrome $P\text{-}450_{cam}$ oxygen complex (stage E, Figure 1) is uncertain. On the basis of the above mentioned modelling studies of stages A, B, and D it would be reasonable to assume that the axial ligand trans to dioxygen is mercaptide. However, Dolphin has recently described low temperature spectra of the combination of reduced iron porphyrins, oxygen, and excess mercaptide in dimethylacetamide containing 5% water (enough to afford equivalent concentrations of thiol) (28). These spectra exhibit a Soret at 476 nm, whereas oxygenated $P\text{-}450_{cam}$ (stage E) is reported to have a normal Soret at 418 nm. Dolphin reports a 1:1 O_2 to Fe stoichiometry from gas absorption measurements on the dilute (10^{-5}M), cold (-45°) solutions. The assignment of this spectrum to that of an oxygen complex is critically dependent on this stoichiometry in view of the facile oxidation of iron(II) and mercaptide and the redox reaction of the ferric thiolate combination shown in eq. 1. Dolphin's results indicate that an axial base different from mercaptide must be present in the oxygenated stage E of the P-450 cycle.

The oxygenated stage E of P-450 exhibits a Mössbauer spectrum different from that of oxyhemoglobin (3). An especially distinctive feature in the Mössbauer spectrum of stage E is the lack of temperature dependence of the quadrupole splitting parameter which is quite temperature dependent in oxyhemoglobin.

We have isolated crystalline dioxygen iron porphyrin complexes having three different modes of axial ligation: imidazole nitrogen (29), tetrahydrofuran oxygen (30), and tetrahydrothiophene sulfur (31). The Mössbauer spectrum of each of these oxygenated complexes differs from the spectrum of P-450 stage E--especially with respect to the temperature dependence of the quadrupole splitting. However, the axial ligand may have little effect on this splitting parameter (32) in which case no good probe will be available to determine the nature of the axial base in stage E.

The thioether dioxygen "picket fence" porphyrin

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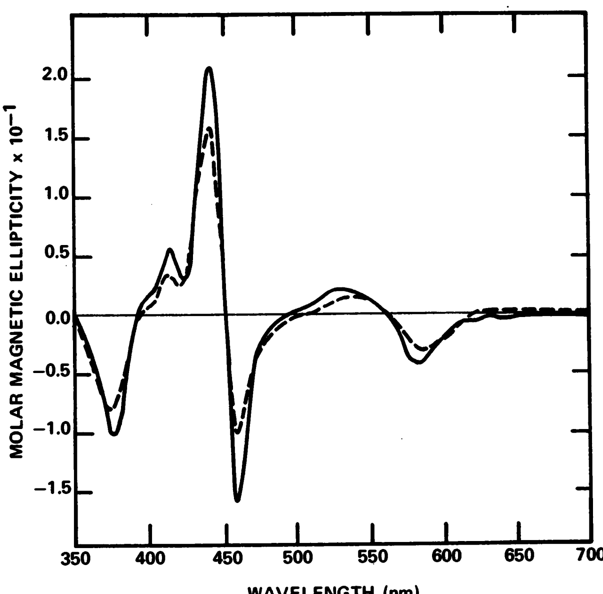

Figure 7. MCD spectra for highly purified P-450$_{LM}$ carbonyl (——) and FePPIXDEE + Na SCH$_3$ + CO in benzene (– – –)

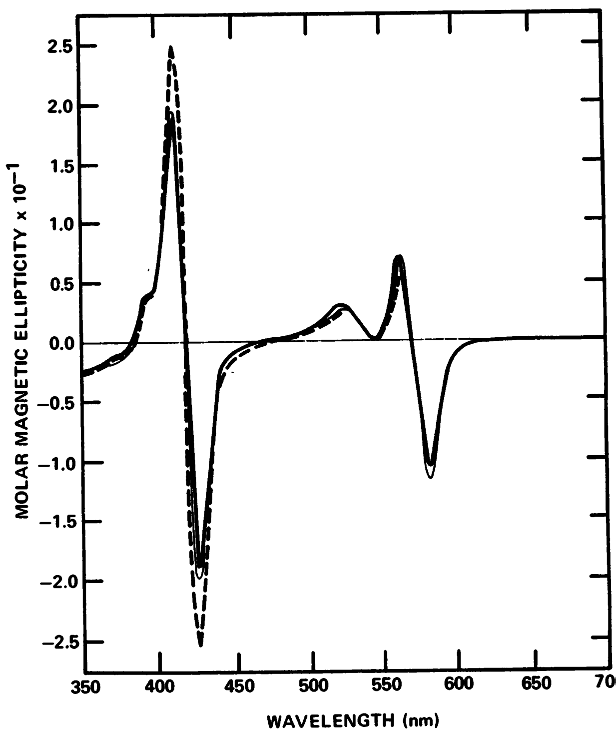

Figure 8. MCD spectra for P-420$_{LM}$ carbonyl (——), FePPIXDEE + N-MeIm + CO in benzene (– – –), and FePPIXDEE + C$_3$H$_7$-SH + CO in benzene (——)

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is relevant to the P-450 models because thioether is expected to have a ligand field character similar to that of thiol (33). The results of an X-ray diffraction study of this complex are illustrated in Figure 9 (31). The structural features of this tetrahydrothiophene complex are similar to those of our earlier N-methylimidazole "picket fence" dioxygen complex except that the terminal oxygen atom does not appear to be disordered in the former and is four-way disordered in the latter. The poor quality of our crystals and the relatively small number of reflections do not permit a sufficiently accurate determination of interesting bond distances to make realistic comparisons.

We have also prepared an oxygen complex from a new type of porphyrin having three "pickets" and a built-in axial thioether base. The synthetic scheme is shown in Figure 10 (31). Oxygenation of cold toluene solutions of this complex leads to slow decomposition but the presence of the oxygen complex has been demonstrated by Mossbauer studies on frozen solutions. However, we have not yet isolated and characterized a stable oxygen complex from this system, although a crystalline carbonyl adduct has been obtained. This system should lend itself to the synthesis and full characterization of model ferrous complexes having axial thiol and thiolate ligands (34). Experiments directed towards this end are in progress. An imidazole derivative of this "three picket" system undergoes reversible oxygenation and we have recently isolated an oxygen complex which is in the process of being fully characterized (35).

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Summary

Model complexes have been generated which accurately reflect spectroscopic features of stages A, B, and D of cytochrome P-450$_{cam}$, although a crystalline model for D has not yet been isolated. The axial ligand common to stages A, B. and D is mercaptide. However the presence of an axial mercaptide ligand in stages C and E is uncertain at present. The reason behind Nature's choice of this unusual ligation is unclear. One possibility has to do with stabilizing the active P-450 oxidant which must be two oxidation levels above the ferric stage. In this stage perhaps one electron is removed from iron affording Fe^{IV}=O and the other from mercaptide generating a thiol radical as suggested by Marchon (36). It is clear that more work must be done in

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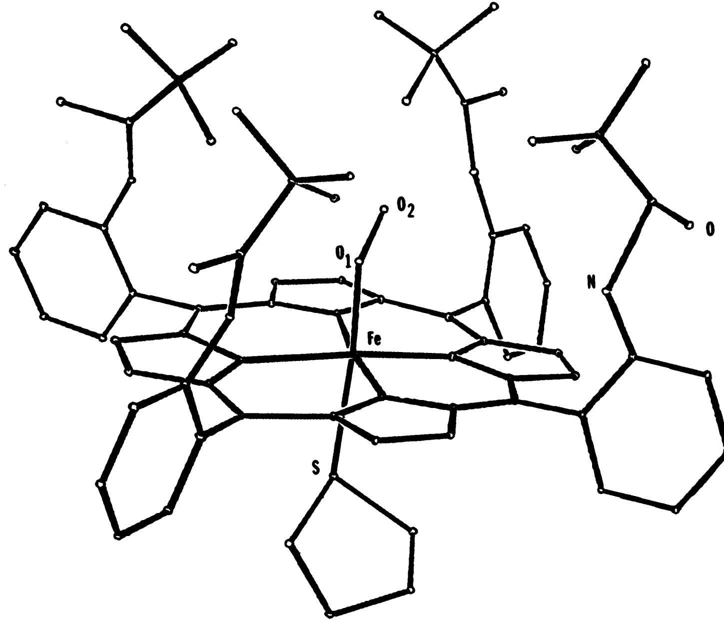

Figure 9. Crystal structure of Fe(TpivPP)(THT)O_2

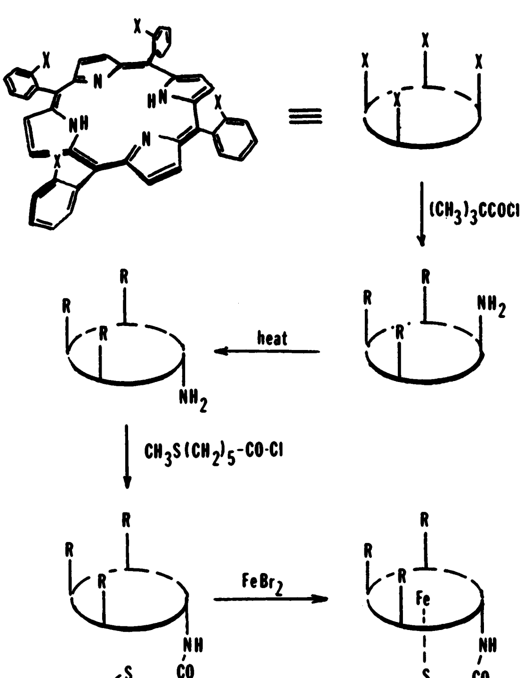

Figure 10. Scheme for preparing "picket fence" porphyrins having an appended axial thioether ligand. X=NH_2 and R=$(CH_3)_3CCONH$–.

synthesizing, isolating, and fully characterizing model porphyrin complexes before we can fully understand the primary coordination spheres employed by cytochrome P-450 in its catalytic cycle.

Abbreviations

P-450:	cytochrome P-450
$P\text{-}450_{cam}$:	cytochrome P-450 camphor hydroxylase
$P\text{-}450_{lm}$:	cytochrome P-450 from liver microsomes
$P\text{-}450_{TBM}$:	cytochrome P-450 from tulip bulb microsomes
TPP:	meso-tetraphenylporphyrin dianion
PPIXDEE:	protoporphyrin IX diethyl ester dianion
PPIXDME:	protoporphyrin IX dimethyl ester dianion
PPIX:	protoporphyrin IX dianion
OEP:	octaethylporphyrin dianion
N-MeIm:	N-methylimidazole
THF:	tetrahydrofuran
THT:	tetrahydrothiophene
(Na)SCH_3:	sodium methyl mercaptide-crown ether complex
DMSO:	dimethylsulfoxide
MCD:	magnetic circular dichroism
esr (or epr):	electron spin (paramagnetic) resonance

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3

Biochemical Studies on Drug Metabolism: Isolation of Multiple Forms of Liver Microsomal Cytochrome P-450

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One of the most interesting properties of the drug-metabolizing enzyme system of liver microsomal membranes is its remarkably broad substrate specificity. Not only drugs and anesthetics, but a variety of other foreign compounds and a number of naturally occurring substances also undergo chemical transformation by this system. Cytochrome P-450, the carbon monoxide-binding pigment of microsomes (1-3), was shown in the pioneering studies of Estabrook and his associates (4-6) to function in the hydroxylation of steroids and the oxidative demethylation and hydroxylation of drugs. Omura and Sato (7) made the important finding that the pigment was a cytochrome of the *b* type and showed that it yielded an altered hemeprotein (cytochrome P-420) when solubilized with phospholipase or deoxycholate. However, hydroxylation activity was lost during the conversion to cytochrome P-420. In 1968 our laboratory reported the resolution of the enzyme system into its components, including a solubilized form of cytochrome P-450 which retained the ability to hydroxylate various substrates (8,9). The present paper will review further studies on the purification and reconstitution of this enzyme system and provide evidence for the occurrence of multiple forms of cytochrome P-450. The existence of a number of distinct forms helps to explain the versatility of this biological catalyst. In addition, evidence supporting the exis-

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tence of two forms of NADPH-cytochrome P-450 reductase differing in apparent minimal molecular weight will be described.

The types of reactions generally attributed to $P\text{-}450_{LM}$[1] are shown in Table I. In addition to hydroxylation (mixed function

TABLE I

Reactions Attributed to Liver Microsomal Cytochrome P-450[a]

Aromatic hydroxylation	N-Oxidation
Aliphatic hydroxylation	Sulfoxidation
N-Dealkylation	Dehalogenation
S-Dealkylation	Azoreduction
O-Dealkylation	Nitroreduction
Deamination	Peroxidation
Desulfuration	Epoxidation

[a]From a review by Gillette (10).

oxidation) reactions, including dealkylations resulting from the hydroxylation of alkyl groups at the carbon atom adjacent to a nitrogen, sulfur, or oxygen atom, the list includes other reactions involving molecular oxygen, such as epoxidation, as well as chemical changes as diverse as dehalogenation and the reduction of nitro and azo groups. A partial list of compounds which serve as substrates for $P\text{-}450_{LM}$ is given in Table II. Included are physiologically important substances as well as drugs and a variety of other foreign compounds. Some of these are taken from an earlier review (10) and no attempt will be made to document this list in view of the vast literature on the subject. It is impossible to estimate accurately the number of substrates attacked by cytochrome P-450 in view of the variety of reactions catalyzed and the fact that newly manufactured xenobiotics of all sorts would be expected to undergo chemical transformation when incubated with this enzyme system. The alteration of drugs by the hepatic endoplasmic reticulum to form products which are inactive or, in some cases, have quantitatively or even qualitatively different activities from the parent compounds, is obviously of great interest to pharmacologists and toxicologists as well as to biochemists.

[1]The abbreviations used are: $P\text{-}450_{LM}$, liver microsomal cytochrome P-450; PB, phenobarbital; BNF, β-naphthoflavone; dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine; SDS, sodium dodecyl sulfate; and PMSF, phenylmethane sulfonyl fluoride.

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TABLE II

Examples of Substrates for Liver Microsomal Cytochrome P-450

Class	Examples
Drugs	Some compounds in each category: narcotics, phenothiazine tranquilizers, anticoagulants, barbiturate sedatives, antidepressants, antihistamines, CNS stimulants, analgesic-antipyretics, sulfonamides, etc.
Anesthetics	Methoxyflurane, fluroxene, etc.
Physiologically occurring compounds	Fatty acids, prostaglandins, steroids (steroidal hormones, cholesterol, and bile acids)
Petroleum products	Aliphatic, alicyclic, and aromatic hydrocarbons
Insecticides	Dieldrin, parathion, etc.
Carcinogens	Polycyclic aromatic hydrocarbons, acetylaminofluorene, etc.

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Resolution and Reconstitution of the Hydroxylation System of Liver Microsomal Membranes

The mixed function oxidase system of liver microsomal membranes was solubilized and resolved in this laboratory into three fractions: A_1, containing a solubilized form of cytochrome P-450; A_2, containing a solubilized form of NADPH-cytochrome P-450 reductase; and B, containing a heat-stable component (8,9). All three fractions were found to be necessary for the hydroxylation of fatty acids, drugs, and various other compounds in the presence of NADPH and molecular oxygen (8,9,11-13). The solubilized cytochrome P-450 retained the properties of the membrane-bound form. For example, it exhibited an electron paramagnetic resonance spectrum typical of a low spin ferric hemeprotein, a carbon monoxide difference spectrum (after chemical or enzymatic reduction) with a maximum at about 450 nm, and typical type I and type II difference spectra with various substrates. Because of the glycerol and other protective agents used during detergent solubilization the cytochrome P-450 preparations contained only low levels of cytochrome P-420. The detergent-solubilized reductase retained the ability of the membrane-bound form to transfer electrons from NADPH to cytochrome P-450 as well as to artificial acceptors such as cytochrome *c*, and could not be replaced in the reconstituted hydroxylation system

by the steapsin-solubilized NADPH-cytochrome c reductase described by Williams and Kamin (14) and kindly furnished by Dr. Kamin.

The heat-stable microsomal factor was shown to have the solubility characteristics of a lipid and was subsequently identified as phosphatidylcholine (15). On the other hand, the microsomal phosphatidylethanolamine fraction was inactive. Dilauroyl-GPC and dioleoyl-GPC were at least as effective as the isolated microsomal phosphatidylcholine fraction, whereas dipalmitoyl-GPC and lysolauroyl-GPC were relatively poor. Both the phospholipid (15,16) and a substrate (17) were required for the rapid phase of reduction of cytochrome P-450 by NADPH in the presence of reductase.

To obtain full activity in the reconstituted enzyme system, the cytochrome P-450, reductase, and sonicated lipid must first be mixed in concentrated form; the buffer and substrate are then added, and the mixture is incubated for several minutes before the final addition of NADPH to initiate the reaction. Small amounts of a detergent, such as deoxycholate, are sometimes also added to ensure maximal activity (18). The dilauroyl compound is used routinely as the phospholipid in hydroxylation assays because it is the most effective and because, unlike the microsomal phosphatidylcholine fraction containing unsaturated fatty acids, it does not readily undergo chemical or enzymatic peroxidation. It should be noted that the hydroxylation activities toward most substrates in the reconstituted enzyme system, expressed as turnover numbers (that is, mol of substrate hydroxylated per mol of P-450_{LM} per min), are at least as great as in microsomal suspensions. In some instances the activities are considerably higher upon reconstitution, since P-450_{LM} can easily be made the rate-limiting component by the addition of saturating levels of reductase and phospholipid, whereas the cytochrome may not necessarily be the rate-limiting component in intact microsomes. The isolated P-450_{LM} and NADPH-cytochrome P-450 reductase individually form small aggregates, and a mixture of the two proteins and the phospholipid behaves similarly. Reconstitution in either the presence or absence of substrate does not cause the formation of very large aggregates or membrane-like structures, as shown by sedimentation studies, gel exclusion chromatography, and electron microscopy (19). We have reported evidence indicating that a mixture of the purified cytochrome P-450 and reductase exists as a complex with an apparent molecular weight of about 500,000 in the presence of phospholipid and substrate (20). By reconstitution of the microsomal system we are referring to the recovery of hydroxylation activity when the individual components are combined in the proper fashion, not to reassembly of the original membrane. The latter task will require greater knowledge of the various other protein, lipid, and carbohydrate components of microsomal membranes.

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Purification and Characterization of Multiple Forms of Liver Microsomal Cytochrome P-450

Experimental Approaches and Proposed Nomenclature. The challenging question of whether the many catalytic activities attributed to P-450_{LM} reside in one or more forms of this cytochrome has been the subject of much investigation. As indicated in several earlier reviews (10,21-23), the various patterns of activities observed in liver microsomes of animals treated with different inducing agents suggest that numerous forms of the cytochrome might be involved. On the other hand, kinetic data obtained with microsomal suspensions (24-26) or with the reconstituted system (13) indicated that a number of substrates act as mutually competitive inhibitors, thereby showing that they may be acted on by a single enzyme. Spectral evidence was reported suggesting the occurrence of two forms of cytochrome P-450 in liver microsomes, induced either by PB or polycyclic aromatic hydrocarbons (27-31), and cyanide titrations of microsomes indicated the possible occurrence of three forms with different binding constants (32). A study of the genetic regulation of aryl hydrocarbon hydroxylation also supported the existence of a separate cytochrome responsible for this activity (33), and it was further shown that substrate specificity resides in the different cytochrome fractions as tested in reconstitued systems (34-36).

Recently, some of the forms of P-450_{LM} have been obtained in a highly purified state, thereby permitting the unequivocal conclusion that these are distinct proteins with different catalytic activities. Our laboratory has reported the isolation of the PB-inducible and BNF-inducible forms of rabbit P-450_{LM} in an electrophoretically homogeneous state (18,37,38) as well as the separation and characterization of other forms with different physical and catalytic properties (38). More recently, as will be described below, we have characterized in detail P-450_{LM_2} and LM_4 isolated from PB- and BNF-induced microsomes, respectively (39), and shown that these proteins also exhibit homogeneity by the Ouchterlony immunochemical technique (20,40). Highly purified P-450_{LM} has also been obtained recently from induced animals by two other laboratories. The isolation of the cytochrome from 3-methylcholanthrene-treated rats and rabbits and from PB-treated rats has been reported by investigators at Hoffmann-La Roche (41,42). Immunochemical studies by the same group (43) showed that P-450_{LM} from the carcinogen-treated rats was probably homogeneous but that the preparation from PB-treated rats contained at least three hemeproteins. The cytochrome has also been isolated in electrophoretically homogeneous form from PB- and 3-methylcholanthrene-treated rabbits by investigators in Japan (44,45). The latter preparation was reported to contain bound carcinogen, thereby accounting for the observed optical spectrum.

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The selection of a suitable nomenclature for the multiple forms of P-450_{LM} is difficult because of our present inadequate knowledge of their properties and functions. Naming these cytochromes by the spectral characteristics of the reduced CO complex (e.g., P-450 and P-448), as frequently seen in the literature, has the serious drawback that several forms have almost identical spectra (38). Furthermore, in no instance do the CO complexes of the cytochromes from rabbit liver absorb exactly at 450 nm. A nomenclature based on the inducer used (e.g., P-450_{PB}, P-450_{BNF}, etc.) appears inadequate at the present time, since several forms of the hemeprotein are not yet known to be inducible. In addition, certain forms which are inducible (as by BNF) are also present in control animals at significant levels. Although it would be logical to name the cytochromes by function, that is, by the substrates attacked, unfortunately the evidence so far available indicates that each of the proteins has a broad spectrum of activities, though with some quantitative differences (38). A further difficulty is that, with the availability of new drugs and other foreign compounds, the picture will gradually change. Until a nomenclature based on the functions of these versatile catalysts becomes possible, it seems advisable to use the electrophoretic method which has proved useful and reproducible (38,39). The proteins are submitted to discontinuous polyacrylamide gel electrophoresis in the presence of SDS by the procedure of Laemmli (46), which in our experience gives the best resolution of the electrophoretic methods presently available. Beginning with the major microsomal band of greatest electrophoretic mobility, the bands are numbered according to decreasing mobility and increasing molecular weight (38), as shown in Figure 1. It can be seen that P-450_{LM_2} clearly corresponds to the protein band induced by PB but absent from normal and BNF-induced microsomes, and P-450_{LM_4} corresponds to the protein band induced by BNF and also present in significant amounts in normal and PB-induced microsomes. In addition, four other forms have been partially purified, and tentatively identified by their specific electrophoretic bands, as will be described below. To illustrate the usefulness of the electrophoretic method of nomenclature, the purified cytochrome from 3-methylcholanthrene-induced rabbits, kindly furnished by Dr. A. Y. H. Lu and his associates, and the purified cytochrome from 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated rabbits (47), kindly furnished by Dr. E. F. Johnson and Dr. U. Muller-Eberhard, are electrophoretically indistinguishable from our BNF-induced P-450_{LM_4}. Accordingly, it can assumed that our three laboratories are working with the same protein and should thus avoid unnecessary confusion by the use of separate names based on the inducer used or the substrates tested by a particular research group. Clearly, it is advisable that forms of cytochrome P-450 isolated from liver microsomes of rabbits treated in various ways, as well as from other organelles, tissues, and species, be exchanged by the various investigators for electrophoretic characterization.

Purification of P-450_{LM_2} and LM_4. The steps used in the purification of P-450_{LM_2} from PB-induced rabbit liver microsomes (18, 37, 39) are summarized in Table III. The microsomes are extracted with pyrophosphate to remove hemoglobin and certain other contaminating proteins. This step is followed by solubilization of the membranes with cholate in the presence of glycerol and other protective agents and fractionation with polyethylene glycol, which precipitates the various forms of P-450_{LM} and leaves NADPH-cytochrome P-450 reductase in the supernatant fraction. Column chromatography of the cytochrome fraction on DEAE-cellulose in the presence of Renex 690, a nonionic detergent, provides further extensive purification. Finally, hydroxylapatite column chromatography gives electrophoretically homogeneous P-450_{LM_2} in yields as high as 9.5% from the starting mixture of various forms of P-450_{LM}. The yield would be considerably higher (perhaps approaching two-fold) if calculated on the basis of the starting P-450_{LM_2}, but the concentration of this form of the cytochrome in microsomes is not

TABLE III

Purification of P-450_{LM_2} from PB-Induced Rabbit Liver Microsomes[a]

Preparation	P-450_{LM} content[b]	Yield
	nmol per mg protein	%
Pyrophosphate-treated microsomes	2.6-4.0	100
Cholate-solubilized preparation; polyethylene glycol precipitate (8 to 10%)	5.4-7.1	40
DEAE-cellulose column eluate (0.1% Renex)	9.0-15.2	24
Hydroxylapatite-silica gel column eluate (0.1% Renex)	13.0-19.7[c]	9.5
Calcium phosphate gel eluate[d]	13.2-20.1	

[a]The detailed procedure is published elsewhere (39).

[b]The values given show the P-450_{LM} content, based on spectral assay of the reduced CO complex, in the various fractions in a series of experiments.

[c]Whereas several forms of the cytochrome are present in previous steps, after hydroxylapatite column chromatography the P-450_{LM_2} preparation is electrophoretically homogeneous.

[d]This step removes excess detergent.

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known accurately. The heme is lost by dissociation to a varying extent, and the preparations are electrophoretically homogeneous after the hydroxylapatite chromatography step but vary in the relative amount of holoenzyme present. Calcium phosphate gel adsorption is usually carried out to remove excess detergent; following this step, the best preparations had a content of 20.1 nmol of holoenzyme per mg of protein (39).

The procedure followed for the purification of P-450_{LM_4} from BNF-induced microsomes uses methods similar to those already described for P-450_{LM_2}. As shown in Table IV, electrophoretically homogeneous P-450_{LM_4} is obtained in yields as high as 9.8% from the starting mixture of various forms of P-450_{LM}; if calculated on the basis of the starting LM_4 in microsomes, the yield would be greater. The best preparations obtained following the calcium phosphate gel adsorption step had 16.8 nmol of P-450_{LM_4} per mg of protein, and the best preparations of P-450_{LM_4} from PB-induced microsomes had 17.2 nmol per mg of protein.

Properties of Purified P-450_{LM_2} from PB-Induced Microsomes and LM_4 from BNF-Induced Microsomes. The subunit molecular weights of P-450_{LM_2} from PB-treated animals and P-450_{LM_4} from BNF-induced ani-

TABLE IV

Purification of P-450_{LM_4} from BNF-Induced Rabbit Liver Microsomes[a]

Preparation	P-450_{LM} content[b]	Yield
	nmol per mg protein	%
Pyrophosphate-treated microsomes	2.3-2.4	100
Polyethylene glycol precipitate	3.2-6.3	61
DEAE-cellulose column eluate	6.1-9.2	16
Hydroxylapatite column chromatography	9.5-14.4[c]	9.8
(Calcium phosphate gel eluate)[d]	(11.1-16.8)[c]	

[a]The detailed procedures are published elsewhere (39).

[b]The values given show the P-450_{LM} content in a series of experiments.

[c]Whereas several forms of the cytochrome are present in previous steps, after hydroxylapatite column chromatography the P-450_{LM_4} preparation is electrophoretically homogeneous.

[d]This step removes excess detergent.

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mals were estimated to be 50,000 and 54,000, respectively, by SDS-polyacrylamide gel electrophoresis with standardization by proteins of known molecular weight (38). Values based on amino acid analysis (kindly carried out by Dr. Karl Dus, Department of Biochemistry, St. Louis University) and allowing for the heme and carbohydrate content are 48,700 for $P\text{-}450_{LM_2}$ and 55,300 for LM_4. These proteins tend to aggregate, and therefore exhibit molecular weights higher than the subunit values under non-denaturing conditions. For example, the apparent molecular weight of solubilized but relatively crude preparations of $P\text{-}450_{LM}$ was estimated by sedimentation velocity measurements, sucrose density gradient centrifugation, and gel exclusion chromatography to be about 350,000 (19), and that of purified $P\text{-}450_{LM_2}$ to be about 270,000 (20). These are in fairly good agreement considering the experimental error in the measurements.

Additional analytical data on these purified proteins are presented in Table V. Heme, determined as the reduced pyridine hemo-

TABLE V

Properties of Purified Cytochromes

Component analyzed	$P\text{-}450_{LM_2}$	$P\text{-}450_{LM_4}$
Cytochrome P-450 (nmol/mg protein)	20	17
Heme (mol/mol protein)	1	1
Carbohydrate (residues/mol protein)	3	3
Amino acids (residues/mol protein)	424	482
Phospholipid (mol/mol protein)	0.3	0.5
Cytochrome b_5, NADPH-cytochrome P-450 reductase, or NADH-cytochrome b_5 reductase	none detected	none detected

chrome (48), is present in an amount not exceeding one mol per mol of protein subunit. Both proteins contain three carbohydrate residues (hexose or hexosamine) and, as expected from the molecular weight differences, $P\text{-}450_{LM_4}$ has about 60 more amino acid residues per polypeptide chain than does $P\text{-}450_{LM_2}$. Following extensive dialysis of the proteins against distilled water to remove phosphate buffer, phospholipids were extracted and estimated by determination of the phosphorus content. The results indicated that during purification the phospholipid content is reduced to very low levels. As shown in the table, several other microsomal enzymes

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could not be detected in these highly purified P-450_{LM} preparations. The Renex 690 concentration in the purified cytochromes was determined by a method developed for nonionic detergents containing polyethoxy groups (49,50); the cobalt ammonothiocyanate complex of the detergent was extracted into chloroform, and the concentration was determined from the absorbance at 322 nm. Earlier studies showed that the detergent concentration in preparations of P-450_{LM_2} partially purified by DEAE-cellulose chromatography was found to be about 0.4 mg per mg of protein (18), whereas the concentration in the highly purified proteins is much less (about 0.05 mg per mg of protein) (39).

Evidence for Additional Forms of P-450_{LM}. In addition to P-450_{LM_2} and LM_4, which have now been isolated and well characterized, as already described, liver microsomes contain hemeproteins other than cytochrome b_5. These were first detected by staining for heme after the preparations were treated with SDS at 5° in the absence of mercaptoethanol (according to the method of Welton and Aust (51)) and submitted to polyacrylamide gel electrophoresis (38). During the purification of P-450_{LM_2} and LM_4, the various fractions were examined for cytochrome P-450 by spectral analysis of the CO complexes and also submitted to gel electrophoresis after treatment with mercaptoethanol and SDS. It soon became evident that forms of P-450_{LM} other than LM_2 and LM_4 were being isolated in other fractions. We have already reported the isolation of a fraction containing P-$450_{LM_{1,7}}$ (a mixture of LM_1 and LM_7) and the further separation of these two forms (38). Such preparations, as well as others which have been designated P-$450_{LM_{3a}}$ and LM_{3b}, all exhibit spectra as the reduced CO complexes typical of cytochrome P-450. P-450_{LM_1}, LM_{3a}, LM_{3b}, and LM_7 have not yet been highly purified or thoroughly characterized, however, and until that has been accomplished the possibility should be considered that these might be electrophoretic artifacts or somehow derived from the two well characterized forms. On the other hand, these four forms do not increase in amount when rabbits are induced by the administration of PB or BNF, as might have been expected if they were derived from P-450_{LM_2} and LM_4. Because of the lack of known inducers, we presently have no way of increasing the amounts of these four forms in order to facilitate their isolation from microsomes. Figure 2 shows all six forms of P-450_{LM} in the 45,000 to 60,000 molecular weight range as seen upon SDS-polyacrylamide gel electrophoresis of the purified samples. For reference, the pattern of polypeptides from PB-induced microsomes is also shown. The bands corresponding to P-$450_{LM_{3a}}$ and LM_{3b} are barely distinguishable in the microsomes, but are more clearly seen to be distinct proteins when the purified samples are compared. As we have already indicated, only P-450_{LM_2} and LM_4 give single electrophoretic bands; LM_1, LM_{3a}, LM_{3b}, and LM_7 have been extensively purified but still contain additional protein components. Accordingly, their designation in this manner by our electrophoretic method of nomenclature should be considered tentative.

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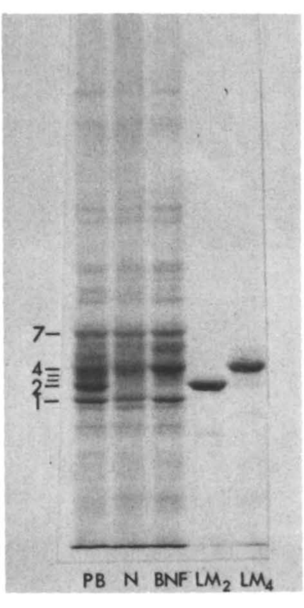

Figure 1. Slab polyacrylamide gel electrophoresis of P-450$_{LM}$ fractions in a discontinuous buffer system. The preparations were treated with mercaptoethanol and SDS at 100° and submitted to electrophoresis according to Laemmli (46) with a 7.5% separating gel. Migration was from top to bottom. The gel was fixed in 65:25:10 water–isopropanol–acetic acid, stained with 0.05% Coomassie Blue in the same solvent, and destained with 80:10:10 water–isopropanol–acetic acid. The following amounts of protein were analyzed: PB-induced, normal (control), and BNF-induced microsomes, 6μg each; highly purified P-450$_{LM_2}$ (from PB-induced microsomes) and P-450$_{LM_4}$ (from BNF-induced microsomes), 1 μg each.

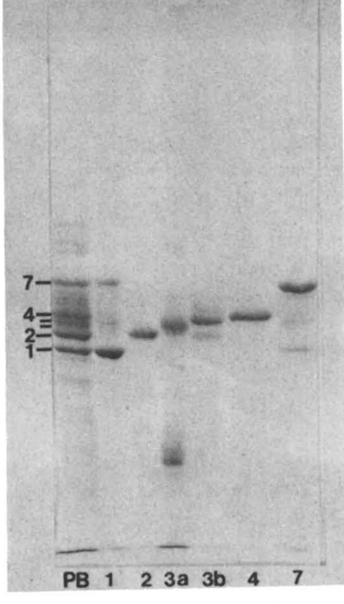

Figure 2. Slab gel electrophoresis of P-450$_{LM}$ fractions. The conditions were as in Figure 1, and the various preparations were as follows with the amounts of protein indicated: PB-induced microsomes (6 μg), LM$_1$ (1.5 μg), LM$_2$ (0.5 μg), LM$_{3a}$ (2.0 μg), LM$_{3b}$ (1.5 μg), LM$_4$ (1.0 μg), and LM$_7$ (1.5 μg).

Immunochemical Studies on Different Forms of Cytochrome P-450. We have also used immunological techniques to compare the properties of various forms of cytochrome P-450. Such studies were begun in collaboration with Dr. Karl M. Dus in order to determine possible similarities between P-450_{cam}, the camphor-hydroxylating cytochrome of *Pseudomonas putida* (52-54), which has been obtained in crystalline form (55), and phenobarbital-inducible rabbit liver microsomal cytochrome P-450, the first such mammalian cytochrome to be obtained in a highly purified state (37). These two cytochromes differ markedly in substrate specificity, solubility, and the requirement of the bacterial cytochrome for an iron-sulfur protein and of P-450_{LM_2} for a phospholipid for hydroxylation activity. Despite these differences, the two highly purified proteins showed immunological cross reaction by radioimmune assay (56,57). Similar techniques have now been used to compare the various forms of P-450_{LM} (40). Rabbits and goats were inoculated with purified rabbit P-450_{LM_2} or LM_4, and it was found that rabbits produced antibodies against P-450_{LM_2} only, whereas goats produced antibodies against both cytochromes. P-450_{LM_2} is present in only trace amounts in uninduced rabbit liver and apparently, therefore, acts as a foreign protein in eliciting an immune response in the same species.

A comparison of the immunochemical properties of some of the forms of P-450_{LM} by Ouchterlony double diffusion analysis is shown in Figure 3. A strong precipitin line was observed in the reaction of anti-P-450_{LM_2} antibodies with PB-induced microsomes or with P-450_{LM_2}, but not with P-450_{LM_4}, P-$450_{LM_{1,7}}$, or control or BNF-induced microsomes. Careful examination of the Ouchterlony plate showed that the precipitin band with P-450_{LM_2} did not extend into the well containing control microsomes, thereby indicating that trace amounts of P-450_{LM_2} are present in uninduced rabbits. In other experiments, competitive binding studies with radiolabeled cytochromes confirmed that rabbit anti-LM_2 does not cross-react with P-450_{LM_4}. On the other hand, slight but significant cross-reactions were detected by this technique between goat anti-LM_2 and P-450_{LM_4}, and between goat anti-LM_4 and P-450_{LM_2}. To summarize, such immunochemical studies show that P-450_{LM_2} and LM_4 have significant structural differences. This conclusion is in accord with other information already presented. In view of the immunochemical differences observed between P-450_{LM_2} and the other rabbit hepatic cytochromes, P-450_{LM_1}, LM_4, and LM_7, it is indeed surprising that the drug-inducible P-450_{LM_2} resembles bacterial P-450_{cam}, as judged both by immunological methods and by amino acid analysis of the heme peptides of these two proteins (56,57).

Comparison of P-450_{LM_4} Preparations from Three Types of Rabbit Liver Microsomes. Since, as already indicated, P-450_{LM_2} is present in only trace amounts in liver microsomes of uninduced animals, one need not be concerned that the cytochrome isolated from PB-treated animals might be a mixture of somewhat different endo-

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genous and induced forms. On the other hand, this possibility exists with P-450_{LM_4}, since this protein is present in about half as great an amount in control animals as in those induced with BNF (cf. Figure 1). To determine whether the endogenous and induced forms of this cytochrome might be electrophoretically similar, but different in other respects, we have recently purified P-450_{LM_4} to homogeneity from BNF-induced, PB-induced, and uninduced animals (39). No differences were seen among these three cytochrome preparations in amino acid content, identity of the C-terminal amino acids, or spectral properties. Indeed, the absolute spectra of the oxidized forms, reduced forms, and CO complexes are indistinguishable both in location of the absorption maxima and in the magnitude of the extinction coefficients. In addition, electrophoretically homogeneous P-450_{LM_4} from the three kinds of microsomes was examined by the Ouchterlony technique, using antisera to the BNF-induced preparation of the cytochrome, and the three preparations exhibited complete identity. The possibility remains, nevertheless, that the various preparations of P-450_{LM_4} differ only slightly, perhaps in a few amino acid residues, and are so similar that they are not distinguished by the techniques so far employed.

Substrate Specificity of Forms of P-450_{LM}. The data in Table VI summarize some of our present information about the catalytic activities of the purified forms of P-450_{LM} toward various sub-

TABLE VI

Substrate Specificity of Different Forms of Cytochrome P-450[a]

Substrate	Position of hydroxyl group in product	Activity of P-450_{LM} preparation				
		LM_1	LM_2	LM_4	$LM_{1,7}$	LM_7
p-Nitrophenetole		2.0	10.5	5.5	2.0	1.0
p-Nitroanisole		2.5	5.7	3.4	3.3	1.8
Benzphetamine		9.6	56.8	4.9	7.7	13.6
Ethylmorphine			6.1	3.0	3.0	
Aniline			1.0	0.4	0.6	
Biphenyl	2		0.7	0.3	0.4	
"	4		5.4	0.4	0.6	
Testosterone	6β		0.02	0.02	0.32	
"	7α		0.02	0.04	0.02	
"	16α		0.43	0.02	0.07	
Benzo(*a*)pyrene			0.04	(Trace)	0.5	

[a] Some of these data are taken from an earlier publication (38). The P-450_{LM_4} was isolated from liver microsomes of BNF-induced rabbits.

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strates. The picture which emerges is that some forms have higher turnover numbers with certain substrates (for example, P-450$_{LM_2}$ with benzphetamine, the 4 position of biphenyl, and the 16α position of testosterone), but that all forms seem to have at least slight activity with all of the substrates. We have also examined the P-450$_{LM_4}$ isolated from microsomes of rabbits treated in various ways for activity toward three different substrates, as shown in Table VII. The results indicate that, within experimental error, the preparations of P-450$_{LM_4}$ from uninduced, PB-induced, and BNF-induced microsomes have the same activity toward the substrates tested. Such results are in accord with other properties of these three preparations, as already discussed, and provide no indication for more than one form of P-450$_{LM_4}$.

TABLE VII

Catalytic Activities of P-450$_{LM_4}$ from Various Sources

Substrate	Source of P-450$_{LM_4}$		
	Uninduced animals	PB-induced animals	BNF-induced animals
p-Nitrophenetole	3.7	4.2	5.3
p-Nitroanisole	2.7	2.2	3.4
Benzphetamine	5.0	3.9	4.9

Purification and Characterization of Liver Microsomal NADPH-cytochrome P-450 Reductase

The enzyme originally called NADPH-cytochrome *c* reductase was initially isolated by Horecker (58) from pig liver acetone powder and was purified from lipase-solubilized liver microsomes by Williams and Kamin (14) and from trypsin-solubilized microsomes by Phillips and Langdon (59). The biological role of this flavoprotein was uncertain, since cytochrome *c* is a normal constituent of mitochondria rather than microsomes. However, it was demonstrated by Ernster and Orrenius (60) that cytochrome P-450, drug-metabolizing activities, and NADPH-cytochrome *c* reductase activity of microsomes were all increased following the administration of drugs to animals. In addition, immunochemical studies established that antibodies prepared against the purified cytochrome *c* reductase were effective inhibitors of microsomal NADPH-linked drug hydroxylations (61). It is now clear from studies with the reconstituted system, utilizing the detergent-solubilized reductase (11,62,63), that cytochrome P-450 is the natural electron acceptor for this flavoprotein. In contrast to the detergent-solubilized flavoprotein, re-

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ductase preparations purified after solubilization with a protease such as bromelain (64) or with a lipase (65) believed to contain a protease (66) do not retain significant activity toward cytochrome P-450 (67). Proteolytic solubilization apparently removes part of the polypeptide chain (68,69), possibly a hydrophobic portion which aids in binding the enzyme to the membrane. As shown by gel filtration studies, the intact (detergent-solubilized) reductase is capable of binding to purified $P\text{-}450_{LM_2}$, whereas the protease-treated reductase apparently is not (20).

We have obtained the detergent-solubilized NADPH-cytochrome P-450 reductase from rat liver microsomes in a highly purified form and shown that it contains both FMN and FAD (62,63). Iyanagi and Mason (70) were the first to report the presence of both flavin coenzymes in purified trypsin-solubilized and partially purified detergent-solubilized reductase preparations. Recently, Dignam and Strobel (71) have obtained an apparently homogeneous reductase preparation from rat liver and have made use of NADP-Sepharose affinity column chromatography to isolate the enzyme in high yield (72). Also, Yasukochi and Masters (73,74) have used 2',5'-ADP-Sepharose affinity column chromatography to isolate the apparently homogeneous enzyme in very good yield.

Specificity of Reductase toward Cytochrome P-450 from Various Sources. It appears that neither the reductase nor the phospholipid confers substrate specificity on the microsomal hydroxylation system, but that such specificity resides in the various forms of the cytochrome, as already discussed. The rat liver reductase fraction (tested earlier with less purified preparations than presently available) and the synthetically prepared dilauroyl-GPC function effectively with various cytochrome P-450 preparations. Thus, cytochrome P-450 from rat (13), rabbit (12), mouse (36), and human liver (75), and from a yeast (*Candida tropicalis*) (76,77) as well as the carcinogen-inducible cytochrome from mouse (36) and rat liver (34) all couple effectively with the rat liver microsomal reductase in the presence of a phospholipid.

Purified Reductase from Rabbit Liver Microsomes. The detergent-solubilized reductase from PB-induced rabbit liver microsomes has been purified in this laboratory as shown in Table VIII. After extraction with pyrophosphate, the microsomes are solubilized with cholate, and this preparation is then treated with polyethylene glycol to precipitate the various forms of $P\text{-}450_{LM}$ (18). Following the removal of polyethylene glycol, the supernatant fraction is submitted to DEAE-cellulose ion exchange column chromatography. The partially purified reductase is subsequently passed over a column of 2',5'-ADP-Agarose [Agarose-bound N^6 (6-aminohexyl)-adenosine 2',5'-diphosphate, obtained from P-L Biochemicals] according to the method used by Yasukochi and Masters (73,74) for the rat and pig liver reductases. The resulting preparation catalyzed the reduction of 60 µmol of cytochrome *c* per min per mg of protein in an assay mixture containing 0.3 *M* potassium phosphate buffer, pH 7.7, at

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TABLE VIII

Purification of NADPH-cytochrome P-450 Reductase from Rabbit Liver Microsomes

Preparation	Specific activity	Yield
	μmol cyt. c reduced per min per mg protein	%
Pyrophosphate-extracted microsomes	0.3-0.6	100
Polyethylene glycol (12%) supernatant fraction	0.6-1.0	60
DEAE-cellulose column chromatography (0.4% Renex)	6.0-12.0	42
2',5'-ADP-Agarose column chromatography (0.1% Renex)	59.8	26

30°. In addition, Figure 4A (sample a) shows that the purified enzyme appears to be homogeneous by SDS-polyacrylamide gel electrophoresis performed according to the method of Laemmli (46). The minimal molecular weight was estimated to be 74,000 by calibrated gel electrophoresis.

When chromatography on ADP-Agarose was replaced by chromatography on DEAE-cellulose in the presence of 0.1% deoxycholate, the partially purified enzyme was further fractionated into two different reductase preparations having specific activities of 44 and 36 μmol cytochrome c reduced per min per mg of protein, respectively. The electrophoretic properties of the former preparation were identical to those of the purified enzyme described above. In contrast, the latter preparation contained as its major constituent a polypeptide with a minimal molecular weight of 68,000 (Fig. 4A, sample b). Both of these enzyme preparations contain FMN and FAD, and both catalyze the reduction of $P\text{-}450_{LM_2}$, LM_4, and $LM_{1,7}$, as determined by NADPH oxidation in a reconstituted system with benzphetamine as the substrate. In all cases the 74,000 molecular weight form was significantly more active than the 68,000 molecular weight form. When either of the reductase preparations was treated with trypsin and then submitted to SDS-polyacrylamide gel electrophoresis, a single major band was seen in the 68,000-molecular weight region, as shown in Figure 4A (sample d). The combined results from the above experiments demonstrate that two distinct forms of NADPH-cytochrome P-450 reductase, differing by 6,000 in apparent molecular weight, may be isolated from rabbit liver microsomes.

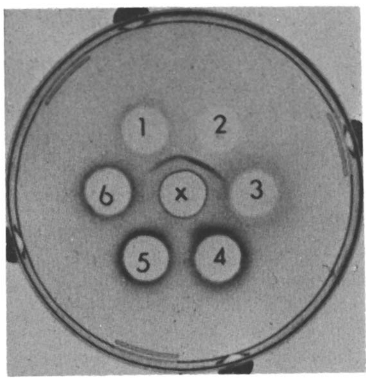
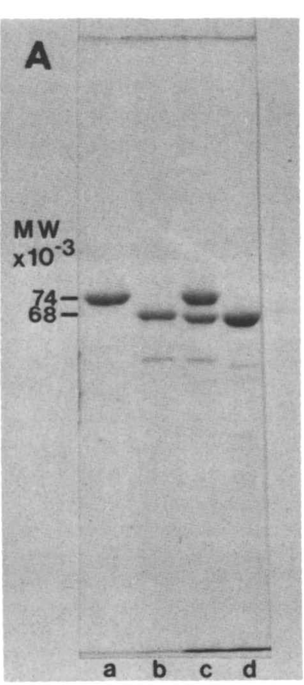


Figure 3. Ouchterlony double diffusion analysis showing reaction of rabbit antibody against P-450_{LM_2} with microsomes or purified cytochrome preparations.

The agar contained 0.5% sodium deoxycholate. The center well contained anti-P-450_{LM_2} γ-globulin (1.5 mg of protein) and the other wells the following: (1) PB-induced rabbit liver microsomes (0.3 nmol of P-450_{LM}); (2) P-450_{LM_2} (0.3 nmol); (3) P-$450_{LM_{1,7}}$ (0.9 nmol of a mixture of about equal amounts of P-450_{LM_1} and $_{LM_7}$); (4) P-450_{LM_4} (0.9 nmol); (5) BNF-induced microsomes (0.9 nmol of P-450_{LM}); (6) control microsomes (0.9 nmol of P-450_{LM}). The plates were incubated at 5° for 24 hr, placed in 1% NaCl containing 0.1% deoxycholate for 24 hr, and then stained with Coomassie Blue (20).



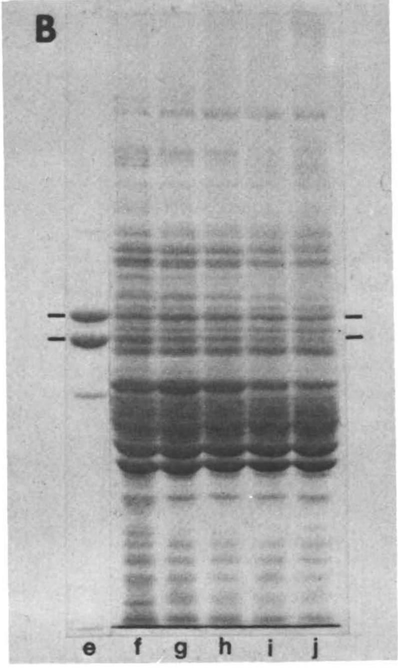


Figure 4. Slab polyacrylamide gel electrophoresis of rabbit liver microsomal NADPH-cytochrome P-450 reductase.

The amount of protein for each sample is indicated in parentheses. (A) purified reductase: a, 74,000 molecular weight form of reductase (0.5 μg); b, 68,000 molecular weight form of reductase (0.5 μg); c, mixture of samples a and b; d, mixture of samples a and b after treatment with trypsin. (B) purified reductase (e same as c above) and pyrophosphate-treated microsomes (f through j, 24 μg each) isolated 3, 6, 9, 13, or 27 hr after a homogenate was prepared from PB-induced rabbit liver. The homogenate was kept at 4° during this time.

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Additional studies were carried out in order to determine if the higher molecular weight form was converted to the lower molecular weight form during the preparation of the microsomal fraction. Figure 4B (samples f through j) shows the results of SDS-polyacrylamide gel electrophoresis of pyrophosphate-extracted microsomes isolated 3,6,9,13, or 27 hr following the preparation of a homogenate from livers of PB-induced rabbits. During this time period, there was only a small decrease in the intensity of the band corresponding to the 74,000 molecular weight reductase and certainly no increase in the intensity of the band corresponding to the 68,000 molecular weight reductase. In fact, the 68,000 molecular weight form of the reductase appears to be more labile, and disappears with a half-life of 21 hours at 4°. This experiment provides no evidence for a direct conversion of the larger form of the reductase to the smaller form during prolonged storage of the liver homogenate at 4°. Preliminary experiments with the protease inhibitor PMSF (78) also suggest that the 68,000 molecular weight form is not a proteolytic artifact produced during purification of the reductases. In a brief report, Satake et al. (69) stated that the molecular weight of the rabbit liver microsomal reductase changed from 91,000 to 84,000 upon treatment with trypsin. The cause for the disparity between their reported molecular weights and those we have determined is not known.

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Purified Reductase from Rat Liver Microsomes. Evidence has also been obtained for the existence of two forms of the reductase in PB-induced rat liver microsomes. We reported previously a procedure for the purification of the detergent-solubilized reductase which leads to the isolation of enzyme purified over 100-fold with respect to the cytochrome c reductase activity of microsomes (62). Table IX summarizes an alternate procedure developed in this laboratory which utilizes Renex-solubilized microsomes (71) and also yields active cytochrome P-450 reductase, but with a slightly higher flavin content and specific activity (79). Enzyme purified by either of these procedures exhibits a single major band when submitted to SDS-polyacrylamide gel electrophoresis. However, as shown in Fig. 5 (samples b,c, and d), the two preparations differ in electrophoretic mobility. Enzyme obtained as described previously (62) has a relative mobility corresponding to a minimal molecular weight of 78,000, whereas that of the enzyme obtained by the procedure outlined in Table IX corresponds to 76,000. Figure 5 (sample a) also shows that microsomes contain two bands with mobilities identical to those of the two preparations of purified enzyme described above.

Although PB-induced microsomes were used as starting material in both purification procedures, the method of drug administration was different. In the earlier work (62), both PB and hydrocortisone were administered via intraperitoneal injection but in the more recent studies (79) animals received PB only in the drinking water. To determine if the isolation of different forms of the re-

TABLE IX

Purification of NADPH-cytochrome P-450 Reductase from Rat Liver Microsomes

Preparation	Specific activity	Yield
	µmol cyt. c reduced per min per mg protein[a]	%
Microsomes	0.4	100
First DEAE-cellulose column eluate (0.5% Renex); calcium phosphate gel eluate	8.6	60
Second DEAE-cellulose column eluate (0.1% deoxycholate); calcium phosphate gel eluate	38.6	37
Third DEAE-cellulose column eluate (0.1% Renex, 0.1% deoxycholate); calcium phosphate gel eluate	61.4[b]	21

[a]Cytochrome c reductase activity was determined at 30° in 0.3 M potassium phosphate, pH 7.7 (59).

[b]The final preparation catalyzed the oxidation of 2.4 µmol of NADPH per min per mg of protein at 30° with rabbit liver $P\text{-}450_{LM_2}$ as the electron acceptor and benzphetamine as the substrate.

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ductase was a result of the use of different induction procedures, liver microsomes were prepared from animals receiving no treatment, or PB pretreatment by either of the two methods described above. These preparations were submitted to SDS-polyacrylamide gel electrophoresis and, as shown in Fig. 6 (samples a through c), two bands of approximately equal intensity were observed in the 76,000 to 78,000 molecular weight region (indicated by the arrow), regardless of the manner in which the animals were treated. These results do not suggest a preferential induction of either band by PB pretreatment, nor does it appear that hydrocortisone alters the relative proportion of these two bands in microsomes.

To examine the possibility that the lower molecular weight form was produced during isolation of the microsomes by proteolytic degradation of the larger polypeptide, homogenization and subcellular fractionation were carried out in the presence of the protease inhibitor PMSF. As shown in Fig. 6 (samples c and d), no significant change in the electrophoretic pattern of microsomes resulted from the use of this inhibitor. The possibility still remains

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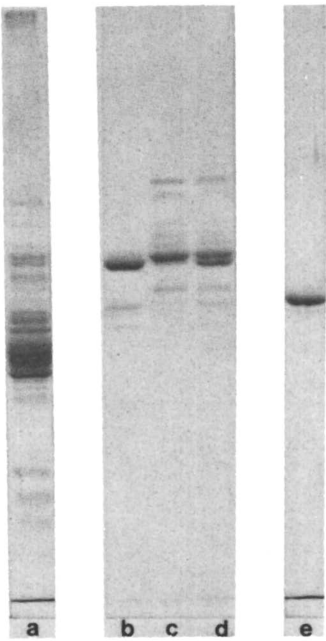

Figure 5. SDS-polyacrylamide gel electrophoresis of PB-induced rat liver microsomes, purified NADPH-cytochrome P-450 reductase, and trypsin-treated reductase as follows.

Sample (a) microsomes, 8 μg of protein; (b) reductase obtained by the procedure summarized in Table IX, 0.7 μg of protein; (c) reductase obtained by the procedure described earlier (62), 1.0 μg of protein; (d) mixture of (a) and (b); (e) mixture of both forms as in (d) following trypsin-treatment, 0.7 μg of protein. Slab gel electrophoresis was performed by a slight modification of the method of Laemmli (46).

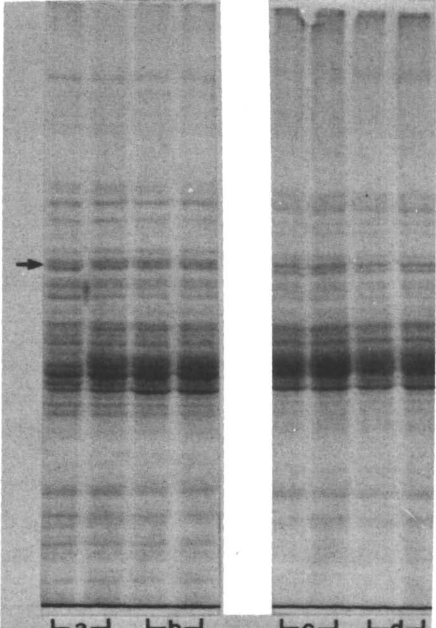

Figure 6. SDS-polyacrylamide gel electrophoresis of microsomes prepared as follows.

(a) From untreated rats, 6 and 8 μg of protein; (b) from rats pretreated with PB and hydrocortisone, 6 and 8 μg of protein; (c) from rats pretreated with PB only in the drinking water, 6 and 8 μg of protein; (d) from rats pretreated as described in (c) but prepared in the presence of the protease inhibitor PMSF, 6 and 8 μg of protein. In the latter case, the homogenizing buffer was supplemented with 0.4mM PMSF, and all other buffers used in the preparation of microsomes contained 0.1mM PMSF.

that a protease insensitive to PMSF may be responsible for the existence of the 76,000 molecular weight form. However, as shown in Fig. 5 (sample *e*), treatment of either form of the purified enzyme with trypsin yields a single large polypeptide with an apparent minimal molecular weight of 69,000 which is significantly less than that of the smaller form of the detergent-solubilized reductase.

These studies are the first to provide evidence for the existence of more than one form of cytochrome P-450 reductase in rat or rabbit liver microsomes. Therefore, the possibility should be considered that there may be discrete pathways of electron transfer involving the selective interaction of a particular form of the reductase with a certain form of P-450_{LM}. More extensive studies on the specificity of the two forms of the reductase for P-450_{LM} are undoubtedly required. Multiple forms of P-450_{LM} with different catalytic activities may explain the broad substrate specificity of the microsomal mixed function oxidase; on the other hand, multiple forms of the reductase may play an important role in determining the overall rates of metabolism of various substrates, particularly under conditions in which the reductase becomes the limiting component in the membrane-bound system.

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Mechanistic Studies with Purified Enzymes

As already indicated, the purified and reconstituted system has been very useful in the study of multiple forms of cytochrome P-450_{LM} and in the elucidation of the role of the phospholipid. It has also proved valuable in studies on the mechanism of the hydroxylation reaction. Only brief mention of such experiments will be made, since they have recently been reviewed elsewhere (80-83), and are not the primary subject of the present paper. In a recent study we have shown that highly purified P-450_{LM_2}, free of other known electron carriers, catalyzes the hydroperoxide-dependent hydroxylation of a variety of substrates in the absence of NADPH, NADPH-cytochrome P-450 reductase, and molecular oxygen (84). The ferrous form of the cytochrome is apparently not involved in such reactions, as indicated by the lack of inhibition by carbon monoxide.

Hydrogen peroxide is formed during substrate hydroxylation in the usual complete reconstituted enzyme system, and must be taken into account in determining the stoichiometry of O_2 and NADPH utilization relative to product formation.[2] Whether peroxide utilization and formation represent the reverse of a common pathway is not yet clear. Evidence has been presented elsewhere that P-450_{LM_2} is a two-electron acceptor and also donates two electrons to molecular oxygen or to artificial electron acceptors (85,86). As shown by stopped flow methods, the reduced protein reacts with molecular oxygen to form two distinct oxyferro complexes (87); Complex I is

[2]Nordblom, G. D., and Coon, M. J., unpublished data.

formed rapidly and then decays to form Complex II which resembles the steady-state intermediate observed by Estabrook et al. (88) with microsomal suspensions. Such studies with the purified cytochrome provide valuable information necessary for understanding the detailed mechanism by which one of the atoms of molecular oxygen is reduced to water by the uptake of two electrons, while the other, presumably as an activated species (most likely oxene bound to the heme iron atom), is inserted into the substrate.

ABSTRACT

The occurrence of multiple forms of rabbit liver microsomal cytochrome P-450 ($P\text{-}450_{LM}$) has been established by the isolation of two forms, phenobarbital-inducible $P\text{-}450_{LM_2}$ and β-napthoflavone-inducible $P\text{-}450_{LM_4}$, in an electrophoretically homogeneous state and by the partial purification of several other forms. The existence of multiple forms of the cytochrome may help to account for the ability of hepatic microsomes to hydroxylate a variety of drugs and other foreign compounds as well as naturally occurring substrates such as steroids and fatty acids. $P\text{-}450_{LM_2}$ and LM_4 differ in their chemical, immunological, and catalytic properties, and are clearly different proteins. NADPH-cytochrome P-450 reductase has also been isolated in an electrophoretically homogeneous state and shown to contain both FMN and FAD. Two forms of the reductase, differing in apparent minimal molecular weight, have been isolated from both rat and rabbit liver microsomes. All of these forms of the reductase retain the ability to transfer electrons from NADPH to $P\text{-}450_{LM}$, as judged by NADPH oxidation in a reconstituted enzyme system with benzphetamine as substrate.

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4

Resolution of Multiple Forms of Rabbit Liver Cytochrome P-450

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Recognition of the existence of multiple forms of cytochrome P-450 is an important step in understanding the complexities of drug and carcinogen metabolism. The characterization of individual forms of cytochrome P-450 is expected to contribute to the knowledge of their function in various metabolic pathways. Investigations undertaken in our laboratory have been directed toward resolving and characterizing forms of cytochrome P-450 from liver microsomes of rabbits treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD. Our efforts have resulted in the partial purification of three distinct forms of cytochrome P-450. These forms possess properties which are unlike those of phenobarbital-inducible cytochrome P-450.

The forms of cytochrome P-450 induced by TCDD, 3-methylcholanthrene, and related inducers are often referred to as cytochrome P-448. These names are derived from differences in the characteristic absorption maximum of the reduced carbonyl cytochrome complex. In addition, the spectra of the cytochromes contrast in the presence of other ligands (1).

The induction of cytochrome P-448 is often linked to increases of several monooxygenase activities. This was extensively examined with inbred mouse strains where a genetic association was found between the induction of a number of monooxygenase activities and mouse liver cytochrome P-448 (2). A prototype activity in this respect is aryl hydrocarbon hydroxylase (benzo(a)pyrene 3-hydroxylase). However, this enzyme is not induced by TCDD or related inducers nor by phenobarbital in liver microsomes of adult rabbits (3-6). Likewise, very few correlate monooxygenase activities are increased. However, acetanilide hydroxylase (6), 2-acetylaminofluorene N-hydroxylase (6), and 7-ethoxyresorufin O-deethylase (7) are increased concomitantly with the induction of rabbit liver cytochrome P-448 by TCDD or 3-methylcholanthrene.

The presence of multiple forms of cytochrome P-450 in rabbit liver microsomes following TCDD treatment might be related to these observations. The form comprising a major portion of

cytochrome P-450 displays a spectrum similar to that observed with microsomal preparations following TCDD treatment. This cytochrome functions in the catalysis of TCDD-inducible monooxygenase reactions. Additional forms of cytochrome P-450 are active in those monooxygenase reactions which are not associated with TCDD- or phenobarbital-induction and are distinguishable from the phenobarbital-inducible cytochrome P-450.

We have employed column chromatography to separate individual forms of cytochrome P-450. Prior to chromatography, liver microsomes from TCDD-treated rabbits were solubilized with sodium cholate and fractionated by polyethylene glycol precipitation as described by van der Hoeven and Coon (8). This method was developed for the isolation of the phenobarbital-inducible form of cytochrome P-450. The cytochromes from TCDD-induced microsomes precipitate over a broad range of polyethylene glycol concentrations. Therefore, fractions containing substantial portions of the partially purified cytochromes were pooled before chromatography (9).

Hydroxylapatite was adopted as the initial separation medium. Two principal fractions of cytochrome P-450 are resolved using a step-wise concentration gradient of potassium phosphate buffer, pH 7.4, containing glycerol (20%), EDTA (0.1 mM), and Nonidet P-40 (0.1%), a nonionic detergent. The two preparations are referred to as cytochrome P-450ab and P-450c (9).

Cytochrome P-450ab represents *ca.* six percent (6%) of the microsomal cytochrome and contains 5.8 nmoles of cytochrome P-450 per mg of protein. The cytochrome P-450c preparation contains *ca.* eighteen percent (18%) of the original cytochrome P-450 pool and 10.0 nmoles per mg protein. One major peptide is detected in the cytochrome P-450c fraction when analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Whereas, several major peptides are observed in cytochrome P-450ab (9).

Immunological differences between the cytochromes were investigated with antiserum produced against the major peptide in the cytochrome P-450c preparation. The major peptide was separated from contaminants by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. A segment of the gel which contained the peptide was used to educe antibody formation in a goat. The antiserum was examined by double diffusion in agarose, and a single precipitin line is seen when it reacts with purified cytochrome P-450c or with sodium cholate-solubilized, liver microsomes from untreated rabbits or rabbits treated with TCDD or phenobarbital. The antiserum to cytochrome P-450c does not cross-react with cytochrome P-450ab (9).

In addition, the antibody was tested for its ability to inhibit microsomal monooxygenase activities. For use in these experiments, an immunoglobulin fraction was isolated from the goat serum by DEAE-cellulose chromatography (10). Experiments recorded in Figure 1 depict the inhibition of microsomal

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acetanilide hydroxylation achieved by addition of increasing amounts of anti-cytochrome P-450c immunoglobulin. A maximum inhibition of *ca.* 80% is observed. Immunoglobulin purified in the same manner from nonimmune goat serum served as a negative control. Neither antibody interfered with the extraction or quantitation of the reaction products. Thus, antibody to the major fraction of cytochrome P-450 (form c) inhibits an activity which is induced by TCDD. Comparable amounts of antibody do not inhibit four monooxygenase activities which are not associated with TCDD-induction. These activities are aryl hydrocarbon hydroxylase (benzo(a)pyrene hydroxylase), aminopyrine N-demethylase, 7-ethoxycoumarin O-deethylase, and coumarin hydroxylase (9).

Two spectral properties of cytochrome P-450c agree with the concept that this form is the TCDD-inducible cytochrome P-448. The Soret maximum of the CO complex of the reduced cytochrome is 447 nm, slightly lower than the value of 449 nm observed for cytochrome P-450ab. In the presence of n-octylamine the oxidized cytochromes exhibit different spectra, as shown in Figure 2. The spectrum of cytochrome P-450c is similar to the n-octylamine difference spectra observed for liver microsomes following induction by TCDD or related inducers. The spectrum of cytochrome P-450ab resembles that of uninduced microsomes (9).

By three criteria, cytochrome P-450ab and P-450c appear to be different forms of cytochrome P-450. They can be chromatographically resolved, are immunologically discrete, and are spectrally distinguishable. Moreover, the specific inhibition of acetanilide hydroxylation by anti-cytochrome P-450c suggests that these cytochromes catalyze different monooxygenase reactions.

However, polyacrylamide gel electrophoresis experiments indicated cytochrome P-450ab might be composed of more than one form of the cytochrome. To further resolve this preparation, DEAE-cellulose was employed. Two fractions were resolved and are referred to as cytochrome P-450a and P-450b (9). Anti-cytochrome P-450c shows no immunological cross-reactivity with either of these forms. DEAE-cellulose is also used to purify cytochrome P-450c to near homogeneity (9).

The isolation scheme is outlined in Figure 3. For comparison, cytochrome P-450 was purified from phenobarbital treated rabbits. The majority of the phenobarbital-inducible cytochrome P-450 was in the fraction corresponding to cytochrome P-450a. Anti-cytochrome P-450c did not cross-react with this cytochrome either. These conditions may not be optimal for purification of this form, but it was obtained with a content of 11.0 nmoles per mg protein and a yield of eighteen percent (18%). It is presumably the cytochrome P-450 LM_2 described by van der Hoeven *et al* (11). However, a direct comparison has not yet been made, and we will denote this form as cytochrome P-450d. The four cytochrome preparations are summarized in Table I.

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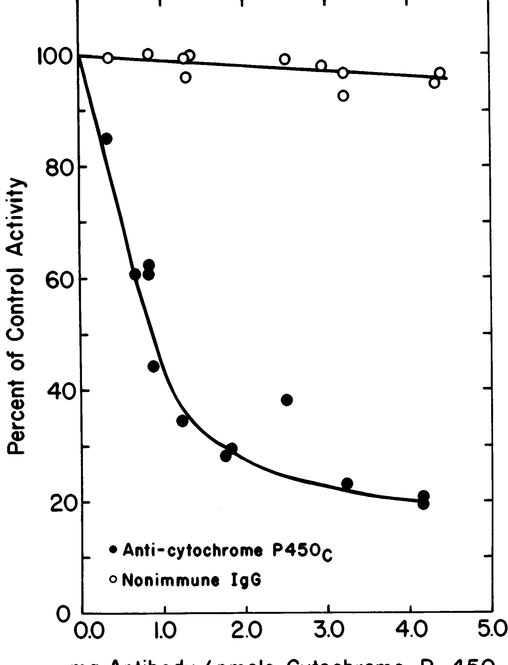

Figure 1. Inhibition of microsomal acetanilide hydroxylase by anti-cytochrome P-450c

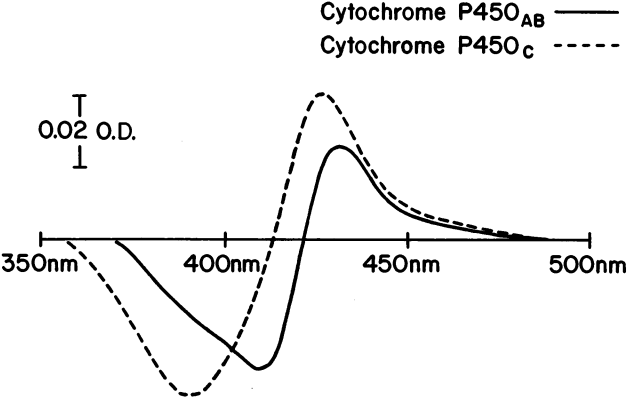

Figure 2. n-*Octylamine difference spectra*

Table I

Summary of Cytochrome P-450 Purification[1]

Cytochrome P-450	Content[2]	Molecular Weight	Yield(%)[3]
a	9.7	48,000 51,000	2
b	12.0	60,000	2
c	17.7	54,500	5
d	11.0	51,000	16

[1]Values are presented for typical preparations

[2]nmoles of cytochrome P-450 per mg protein

[3]based on total microsomal cytochrome P-450

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The four forms of cytochrome P-450 appear to consist of peptides of different molecular weight. These have been determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Results of these experiments are shown in Figure 4 and are presented in Table I. With the exception of cytochrome P-450c, the cytochromes seem to be associated with a single major electrophoretic band. Cytochrome P-450a comprises two principle peptides; one of these corresponds to the phenobarbital-inducible form.

The four cytochromes catalyze different monooxygenase reactions when reconstituted with NADPH cytochrome P-450 reductase. For these experiments, highly purified reductase is routinely isolated from rabbit liver microsomes. We use a slight modification of the affinity chromatographic procedure described by Yasukochi and Masters (12). These reductase preparations catalyze the reduction of 42-55 nmoles of cytochrome c/min/mg protein and are obtained with 38% to 55% overall yields. In addition, the phospholipid dilauroyl-L-α-lecithin is included in the assay mixture. The activity of the individual cytochrome P-450 preparations with a variety of substrates is shown in Table II.

When reconstituted, the four cytochromes show clear differences in their metabolism of the substrates we have tested thus far. Cytochrome P-450d rapidly N-demethylates benzphetamine which is in accord with our suggestion that this is the phenobarbital-inducible form described by van der Hoeven *et al* (11). In addition, cytochrome P-450c actively hydroxylates acetanilide. This was expected as this form of the cytochrome and this

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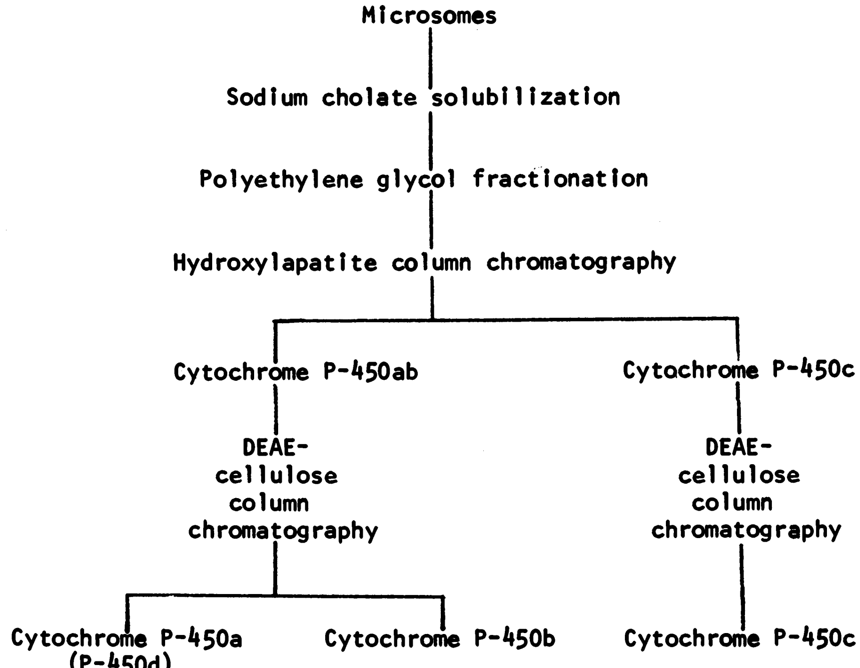

Figure 3. Schematic of the resolution of multiple forms of cytochrome P-450

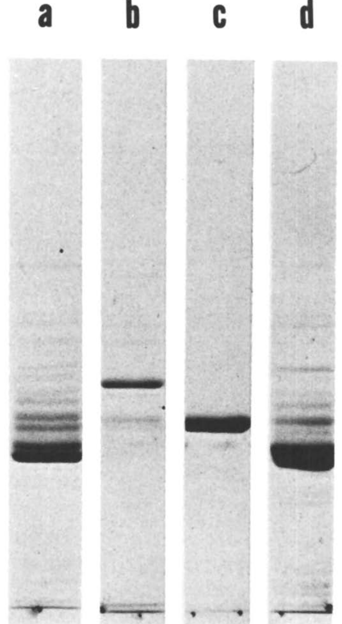

Figure 4. Polyacrylamide gel electrophoresis of four forms of cytochrome P-450 (8 μg) in the presence of sodium dodecylsulfate

TABLE II

RECONSTITUTED ENZYME ACTIVITIES

Substrate	Cytochrome P-450a	P-450b	P-450c	P-450d
Benzphetamine	12	4.0	2.0	51
Benzo(a)pyrene	0.42	4.1	0.03	0.03
7-ethoxyresorufin	0.04	0.4	0.4	0.003
Acetanilide	1.2	1.3	6.1	1.1

Activities are expressed as moles of product formed per mole of cytochrome P-450. NADPH cytochrome P-450 reductase was present in excess to ensure that cytochrome P-450 was the rate limiting component. Reactions were performed in a total volume of 1 ml containing 0.05 M Hepes buffer, pH 7.4 and dilauroyl-L-α-lecithin (30 μg). $MgCl_2$ (15 μmoles) is present when benzphetamine and benzo(a)pyrene are the substrates tested. Products of each substrate were quantitated as described by the following authors: benzphetamine as HCO, T. Nash (1953) Biochem. J. 55, 416-421; benzo(a)pyrene as 3-hydroxybenzo(a)pyrene, D. Nebert and H. Gelboin (1968) J. Biol. Chem. 243, 6242-6249; 7-ethoxyresorufin as resorufin, M. Burke and R. Mayer (1974) Drug Metab. Dispos. 2, 245-253; acetanilide as hydroxyacetanilide, K. Krisch and H. Staudinger (1961), Biochem. Z. 334, 312-327. Reactions were initiated by addition of NADPH (0.4 μmoles).

activity are both induced by TCDD, and the microsomal enzyme is inhibited by anti-cytochrome P-450c.

Benzo(a)pyrene is metabolized rapidly by cytochrome P-450b. The turnover value observed with this cytocrome (9) is comparable to that reported for a highly purified preparation of rat liver cytochrome P-448 (13). Both the phenobarbital- and the TCDD-inducible forms (d and c) are much less active with this substrate as assayed by formation of fluorescent phenols. Thus, rabbit liver does appear to contain an active aryl hydrocarbon hydroxylase (9).

The results we have presented are consistent with the presence of multiple forms of cytochrome P-450 in liver microsomes of rabbits treated with TCDD. The major form, cytochrome P-450c has been purified to near homogeneity and has spectral properties ascribed to cytochrome P-448. However, it does not actively hydroxylate benzo(a)pyrene when reconstituted with

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NADPH-cytochrome P-450 reductase. This form of the cytochrome is immunologically distinguishable from the other forms we have described.

Aryl hydrocarbon hydroxylase (benzo(a)pyrene hydroxylase) was reconstituted with cytochrome P-450b. This second form of the cytochrome has been extensively purified and appears to be composed of a peptide of higher molecular weight than either cytochrome P-450c or cytochrome P-450d.

The third form, cytochrome P-450a, may be a mixture of several cytochromes. Although it is isolated in a manner similar to the isolation of the phenobarbital-inducible cytochrome, form d, it metabolizes benzphetamine at a slower rate. None of the substrates tested thus far is rapidly metabolized by this cytochrome. Polyacrylamide gel electrophoresis experiments show that this preparation consists of several peptides with different mobilities. Two prominent bands are observed. One band comigrates with cytochrome P-450d, and the presence of this cytochrome in the preparation may account for the benzphetamine N-demethylase activity observed. The other major band has a faster mobility than those of the other three cytochrome preparations.

Several investigators have described multiple forms of cytochrome P-450. These include two highly purified forms isolated from rat liver microsomes (12) and several forms isolated from mouse liver microsomes (14). Several forms have also been resolved from uninduced (15) and phenobarbital-induced (16) rabbit liver microsomes. The number of discrete forms of cytochrome P-450 present in rabbit liver microsomes is uncertain, and the correspondence between preparations isolated in various laboratories is not yet clear.

In order to begin a clarification of this situation, a comparison was made between cytochrome P-450 LM_4 isolated from β-naphthaflavone induced rabbit liver microsomes (16), cytochrome P-448 isolated from 3-methylcholanthrene induced rabbit liver microsomes (17), and our cytochrome P-450c. TCDD, β-naphthaflavone, and 3-methylcholanthrene are related inducers of cytochrome P-450. These three cytochrome P-450 preparations react strongly with antibody prepared against cytochrome P-450 LM_4 or P-450c. They also exhibit identical mobilities in polyacrylamide gel electrophoresis experiments. These experiments were performed in Dr. M.J. Coon's and our laboratories.

The work we have described indicates that benzphetamine N-demethylase, benzo(a)pyrene 3-hydroxylase, and acetanilide hydroxylase are each associated with distinct forms of cytochrome P-450. The demonstration of substrate specificities for multiple forms of cytochrome P-450 is an important aspect of this investigation and indicates each form may function in different metabolic pathways. In addition, it may be possible to use these or other assays to detect individual cytochromes in tissue preparations.

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Enantiomeric Selectivity and Perturbation of Product Ratios as Methods for Studying the Multiplicity of Microsomal Enzymes

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It is now well established that the process of the biotransformation of ingested exogenous substances may form reactive electrophilic intermediates which in turn may be responsible for various kinds of potentially lethal toxicities including carcinogenesis. Covalent binding of such intermediates to nucleophilic sites in critical catalytic and structural proteins or nucleic acids is being recognized as one of the major molecular mechanisms for the manifestation of such toxicities. As a consequence, intensified investigations have been initiated to delineate and categorize the multiple forms of the enzymes present in the endoplasmic reticulum of mammalian tissues since these enzymes are largely responsible for such reactions. Intimate knowledge of the specific, catalytic and structural spectrum of the system would at the very least alert society to the real scope of the problem and would undoubtedly suggest methods by which potential toxic hazards could be recognized and therefore be either circumvented or corrected.

The suggestion that more than one enzyme system was responsible for the oxidations of drugs was made almost two decades ago (1,2) and was based on the differential induction of drug metabolism by phenobarbital and polycyclic aromatic hydrocarbons. Subsequent studies directed towards the elucidation of the multiplicity of the microsomal cytochrome P-450 enzymes have segregated along two main lines of investigation, direct and indirect. Direct studies are the most recent and are largely biochemical in nature. Investigators employing such studies have focused on the isolation, purification, characterization and reconstitution of both the normal enzymes and various inducible forms from several different species. Levin and Lu et al. (3-11) utilizing chromatographic electrophoretic and immunologic techniques have succeeded in separating and purifying the microsomal cytochrome P-450's from both rat and rabbit liver after induction with either phenobarbital (PB) or 3-methylcholanthrene (3-MC). Similarly Coon et al. (12-15) have isolated and characterized multiple forms of cytochrome P-450 as have Aust et al. (16,17).

Indirect studies are much more numerous and have arrived at the conclusion of the multiple nature of the cytochrome P-450's present in crude microsomal preparations by inference. Generally differences produced in the catalytic profiles of the system produced by some perturbation are interpreted as evidence for multiplicity. Typically changes produced by either inducing agents (1,2,19-27) or inhibitors (28,29) are studied and often conclusions regarding multiplicity are buried within the manuscript since the original intent of the study was focused on some other question (27,30,31). Two recent examples from the literature illustrate the basis of the indirect approach. Selander, Jerina and Daly (32) studied changes in the ratios of the *o*, *m* and *p*-chlorophenol metabolites of chlorobenzene produced by the hemoprotein-monoxygenase system at various stages of resolution. In addition they studied the differential effects produced by perturbations such as the inducing agents PB and 3-MC, the inhibitors, carbon monoxide, metyrapone, glutathione, SKF-525a and 7,8-benzoflavone and changes in the dependence of product rate on substrate concentration. These differential effects allowed the authors to conclude that at least three cytochrome P-450's differing in regioselectivity and operating by two distinct mechanisms were involved in the hydroxylation of chlorobenzene. Burke and Bridges (33) in a conceptually analogous approach studied changes in the ratios of the 2- and 4-hydroxybiphenyl metabolites of biphenyl produced by a range of perturbations. These authors concluded that at least two independent enzyme systems must be responsible in order to account for the data.

It is somewhat surprising that in the wealth of different perturbations such as inducers, inhibitors, age, sex, species, aging of microsomes, different preparations, etc., that have been used as tools to probe the multiplicity of the microsomal system, stereochemical factors have been essentially ignored. The remainder of this chapter will focus on work done in our laboratories (Sprague-Dawley rats) and the New York State Department of Health (Wistar rats) on the use of stereochemical factors for such studies and the introduction and initial development of a systematic framework for the interpretation of such data.

The oral anticoagulant warfarin, **1**, exists in two enantiomeric forms. In both man (34,35) and the rat (36-38) the *S*-enantiomer is five to six times as potent as the *R*-enantiomer. Moreover, it is known that the drug is stereoselectively metabolized both quantitatively and qualitatively in man (39) and that the metabolic patterns are quantitatively affected by prior administration of other drugs (40,41). In the rat the metabolic patterns both *in vivo* (42) and *in vitro* (43) had been reported but the metabolic patterns of the individual enantiomers were reported only recently (44,45). Since the drug is metabolized to five isomeric hydroxylated products it is well suited for probing the microsomal system from the viewpoint of

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stereochemical factors in order to explore both the phenomenon of drug interactions and multiplicity.

The Michaelis-Menten kinetic parameters for the formation of the individual hydroxylated metabolites from each of the enantiomers are given in Table I and Figure 1. In the analysis to follow the results obtained from each of the enantiomers will be discussed separately and then in combination.

Table I. Comparative Kinetics of the Oxidation of R and S Warfarin by Normal Microsomes From Rat Liver (Sprague Dawley)

Warfarin Metabolite	Apparent Km(mM)*	Apparent V_{max} (nmoles/mg protein, 10 min incubations*)	
	(R)	(R)	d·f
6-hydroxy	0.096±0.028	0.754±0.049	22
7-hydroxy	0.046±0.024	1.653±0.113	22
8-hydroxy	0.093±0.051	0.448±0.052	22
4'-hydroxy	0.109±0.038	0.500±0.042	19
benzylic hydroxy	0.803±0.400	1.693±0.527	19
	(S)	(S)	
6-hydroxy	0.032±0.011	0.644±0.023	23
7-hydroxy	0.050±0.010	0.455±0.013	23
8-hydroxy	0.198±0.048	0.200±0.015	19
4'-hydroxy	0.067±0.021	0.652±0.036	23
benzylic hydroxy	0.221±0.157	0.236±0.060	19

*Data were derived from weighted least-squares linear regression of $[S]/_v$ vs. [S]. The data is expressed as the means ± standard errors with degrees of freedom (d·f) as shown.

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Hydroxylation of R-Warfarin

The apparent Km for the 6-, 7- and 8-hydroxylation of R warfarin are statistically indistinguishable at the 95% confidence level. Since a single substrate is being transformed in a chemically and spatially discrete part of the molecule (coumarin ring) into three structurally distinct products, it is reasonable to assume based on this evidence alone that the products are formed at a single enzymatic site and that product formation is rate limiting. Similarly the Km for 4'-hydroxylation is indistinguishable from those of coumarin ring hydroxylation. Hence this line of evidence is consistent with a single hemoprotein being responsible for all the aromatic hydroxylated products obtained from R-warfarin and the observed differences in V_{max} reflect differences in the various activation energies for product formation irrespective of mechanism.

Although the Km for benzylic hydroxylation, Table I, appears to be larger than the others, the imprecision of its determination

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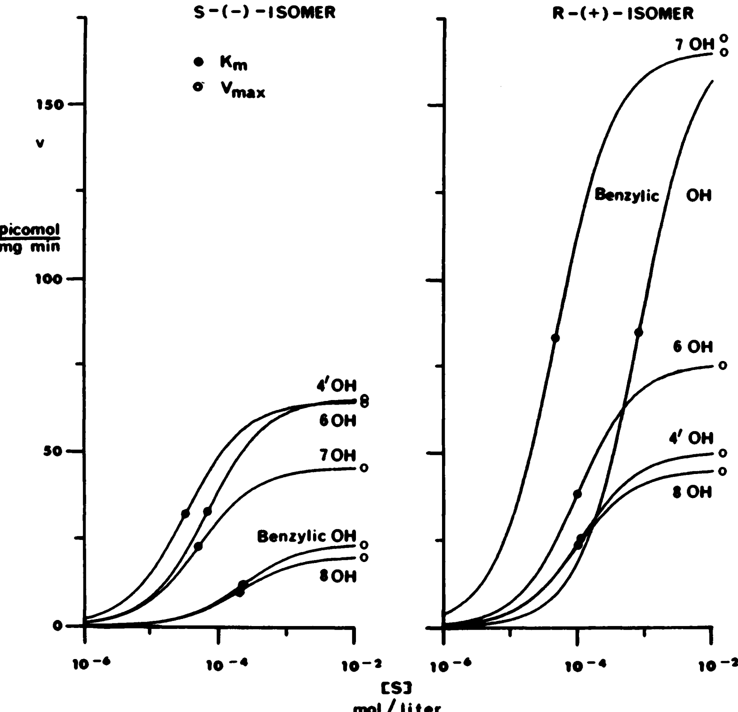

Figure 1. The theoretical velocity for the formation of each of the hydroxylated products was calculated from the experimentally determined Km *and* V_{max} *values and plotted as a function of log [S]. Such plots approach* V_{max} *asymptotically and have inflection point [S] =* Km.

(due to the lack of a synthetic standard) does not allow it to be distinguished statistically. However, other evidence suggests that the Km for benzylic hydroxylation is larger than the Km for any of the aromatic hydroxylations. For example, a smaller quantity of the benzylic hydroxy metabolite was obtained in a single concentration study relative to the quantities of the aromatic hydroxylation products (44) but the V_{max} for benzylic hydroxylation is greater than the V_{max} for either 4'- or 8-hydroxylation. This can only be true if the Km for benzylic hydroxylation is indeed larger than the Km for either 4'- or 8-hydroxylation. The fact that the Km for 6- or 7-hydroxylations are no larger than the Km for 4'- or 8-hydroxylation implies that the Km for benzylic hydroxylation must be larger than the Km for any of the aromatic hydroxylation processes. This result is perhaps not too surprising since benzylic hydroxylation results from a fundamentally different chemical reaction which involves oxidation at a sterically hindered aliphatic site.

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Hydroxylation of S Warfarin

Unlike the results obtained for R-warfarin the Km for 8-hydroxylation from S-warfarin is significantly larger than the Km for either 6- or 7-hydroxylation. Thus, it is clear that two kinetically distinct enzymatic processes must be involved despite the proximity of the sites of metabolic attack. If product formation is rate limiting the contribution of the rate constant for product formation to Km should be negligible; hence, all the Km should be the same while the V_{max} may be different. If product formation is not rate limiting but a common intermediate is involved, two cases are possible; the rate of formation is comparable to the rate of dissociation of the common intermediate back to substrate or the rate of formation of the common intermediate itself may be rate limiting. In the first case the product which is formed with the greatest V_{max} must also have the greatest Km while in the second case the Km for all the products must be the same. Since 8-hydroxylation occurs with the largest Km coupled to the smallest V_{max}, none of the above conditions are fulfilled. Hence, there can be no common intermediate in the formation of 8-hydroxywarfarin and either 6- or 7-hydroxy metabolites.

As was the case for R-warfarin, the Km for 4'- and benzylic hydroxylation alone yield little new information. Although the evidence is less convincing in this case, the data suggests that Km for benzylic hydroxylation is larger than the rest.

Comparative Hydroxylation of the Enantiomers

A high degree of stereoselectivity as evidenced by the differing V_{max} values for each of the enantiomeric pair of products is displayed. The indistinguishability of the Km

for 6- and 7-hydroxylation of a given enantiomer suggests on first analysis that a common enzymatic site is responsible for the generation of these two products. Since arene oxides are well established intermediates in aromatic hydroxylation processes by microsomal mixed function oxidases (45), the possible intermediacy of a 6,7-epoxide in their formation is an attractive initial Postulate. However several lines of evidence argue against this possibility. When the Km for the two 6-hydroxylation products are compared they are found to just differ at the 95% confidence level. This is not true for 7-hydroxylation. If 6- and 7-hydroxylation occur via the intermediacy of an epoxide, a stereoselective difference in Km for the 6-hydroxy metabolites should be reflected in a corresponding identical difference in the Km for the 7-hydroxy metabolites. This does not appear to be the case if the Km differences are real. Studies of the aromatizations of arene oxides have shown that the rate-limiting step of this process involves the spontaneous opening of the arene oxide. The direction in which the opening occurs depends on the stability of the carbocationoid transition state (47-54). In the case of the postulated arene oxide this would imply that ring opening should occur to yield primarily the 6-hydroxylated product because of the ortho and para directing effect of the coumarin lactone oxygen. However, since twice as much 7- as 6-hydroxy product is obtained from R-warfarin it is reasonable to conclude that an epoxide is probably not involved in the formation of the 6- and 7-hydroxy metabolites from this isomer.

Similar electronic arguments permit one to conclude that a 7,8-epoxide cannot be an intermediate in the 7-hydroxylation of R-warfarin while the non involvement of a common intermediate in the formation of the 7- and 8-hydroxy metabolites of S-warfarin is based on the kinetic arguments presented earlier. Therefore, if 7,8-epoxidation occurs in the metabolism of either isomer it must yield 8-hydroxywarfarin almost exclusively.

Unlike coumarin hydroxylation, 4'-hydroxylation is stereospecific for the S-enantiomer but these results are consistent with either a single or multi-enzyme system. The apparently larger Km for benzylic hydroxylation of both isomers suggests that if product formation is rate limiting, aliphatic hydroxylation is probably the result of a distinct microsomal enzyme. Thus, the data is consistent with involvement of at least three kinetically distinct enzymes or enzymatic sites. One enzyme would be responsible primarily for the oxidation of S-warfarin to 8-hydroxywarfarin. A second could be responsible for all the remaining phenolic products and a third for the formation of benzylic hydroxywarfarin.

To further probe the system the studies were repeated at a single concentration after pretreatment of the animals with either PB or 3-MC (55). The results of this study are shown in Table II (Sprague-Dawley), Table III (Wistar) and Figure 2. The data can most readily be analyzed in terms of the following

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Table II. In vivo Comparative Oxidation of R- and S- Warfarin by Normal, PB and 3-MC Induced Hepatic Microsomes from Sprague-Dawley Rats.

Warfarin Metabolites	Product (nmoles/mg protein/10 min*)		
	Normal	PB	3-MC
	(R)	(R)	(R)
6-hydroxy	0.69±0.05	1.35±0.04	2.78±0.17
7-hydroxy	1.92±0.13	4.36±0.26	0.94±0.13
8-hydroxy	0.43±0.02	0.92±0.04	4.91±0.09
4'-hydroxy	0.38±0.02	0.89±0.03	0.16±0.02
benzylic hydroxy	0.26±0.03	0.40±0.01	0.09±0.01
Total	3.68	7.92	8.88
	(S)	(S)	(S)
6-hydroxy	0.64±0.02	1.23±0.07	1.70±0.06
7-hydroxy	0.43±0.01	1.70±0.05	0.40±0.02
8-hydroxy	0.13±0.01	0.42±0.02	0.54±0.03
4'-hydroxy	0.64±0.04	1.12±0.12	0.27±0.04
benzylic hydroxy	0.11±0.01	0.94±0.04	0.09±0.01
Total	1.95	5.41	3.00

*The data is expressed as the means ± standard deviations and represents three analyses. A warfarin concentration of 0.26 mM was employed in these studies.

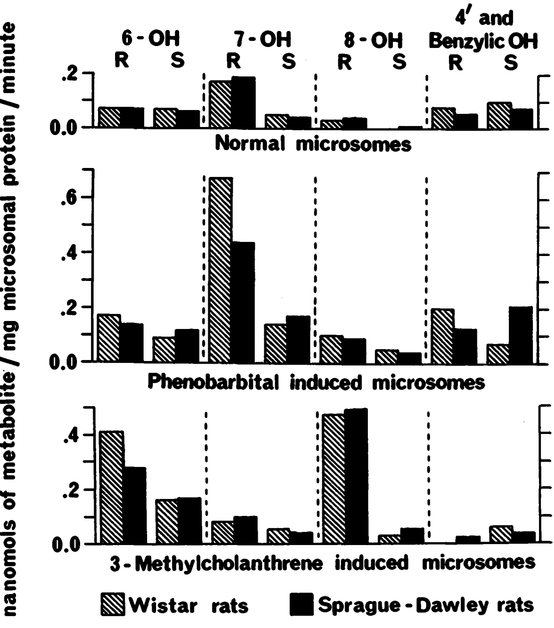

Figure 2. Quantitative microsomal metabolism of the enantiomers of warfarin by normal-, PB-, and 3-MC-induced animals

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Table III. In vivo Comparative Oxidation of R- and S-Warfarin by Normal, PB and 3-MC Induced Hepatic Microsomes from Wistar Rats.

Warfarin Metabolites	Product (nmoles/mg protein/20 min*)		
	Normal	PB	3-MC
	(R)	(R)	(R)
6-hydroxy	1.36±0.12	3.41±0.61	8.16±0.23
7-hydroxy	3.31±0.18	13.41±1.09	1.65±0.41
8-hydroxy	0.59±0.12	1.95±0.28	9.30±1.46
4'-hydroxy plus benzylic hydroxy+	1.54±0.02	3.90±0.95	0.10±0.10
Total	6.80	22.67	19.11
	(S)	(S)	(S)
6-hydroxy	1.30±0.37	1.83±0.07	3.20±0.36
7-hydroxy	1.00±0.12	2.80±0.56	0.92±0.15
8-hydroxy	0.10±0.10	0.97±0.37	0.64±0.09
4'-hydroxy plus benzylic hydroxy	2.01±0.25	1.46±0.42	1.28±0.48
Total	4.31	7.06	6.04

*The data are expressed as the means ± standard deviations and represent three analyses. A warfarin concentration of 0.60 mM was employed in these studies.
+4'-Hydroxy and benzylic hydroxy were not separately quantitated.

regiospecific hydroxylation pathways: 6- and 8-hydroxylation, 7-hydroxylation and 4'- and benzylic hydroxylation.

6- and 8-Hydroxylation

It has been suggested that induction by 3-MC merely alters the relative proportions of multiple forms of cytochrome P-450 (17,56-58). If the results from normal rats are considered as a control and are subtracted from the 3-MC results, the residual quantities must be due to the 3-MC enzyme(s). When the data are viewed in this way it is clear that the 3-MC inducible enzyme(s) are stereoselective for the R-enantiomer and regioselective for 8-hydroxylation of the R-enantiomer and 6-hydroxylation of the S-enantiomer. If the results for the R and S enantiomers are summed the apparent regioselectivity is greatly reduced thus emphasizing the utility of enantiomers as probes of microsomal systems.

If the PB induction data are considered in the same manner, the PB induced enzyme(s) are found to be both less stereoselective and less regiospecific.

The stereoselectivity and regiospecificity observed for normal enzymes are intermediate to that observed for 3-MC and

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PB induced microsomes. These data, on first analysis, are therefore consistent with the postulate that normal microsomes are comprised of a mixture of 3-MC and PB inducible systems. However, the contribution of the 3-MC inducible enzyme(s) must be minimal since the stereoselectivity of the normal enzymes for 8-hydroxylation are close to that displayed by the PB induced system and different from the 3-MC system, Figure 3. Moreover, the regioselectivity of the 3-MC system for 8-hydroxylation of the R isomer is inconsistent with the preferential formation of the 6-hydroxy metabolite in the normal system. Thus, the results are consistent with the 6- and 8-hydroxylase activity of normal microsomes being comprised primarily of PB inducible enzyme(s). The possibility of a minor contribution from 3-MC and/or non inducible enzyme(s) cannot be excluded.

7-Hydroxylation

The enzymatic processes responsible for the formation of 7-hydroxywarfarin can be distinguished from coumarin 6- and 8-hydroxylase(s) on the observed differences in stereoselectivity produced in both strains of rats by induction with 3-MC or PB

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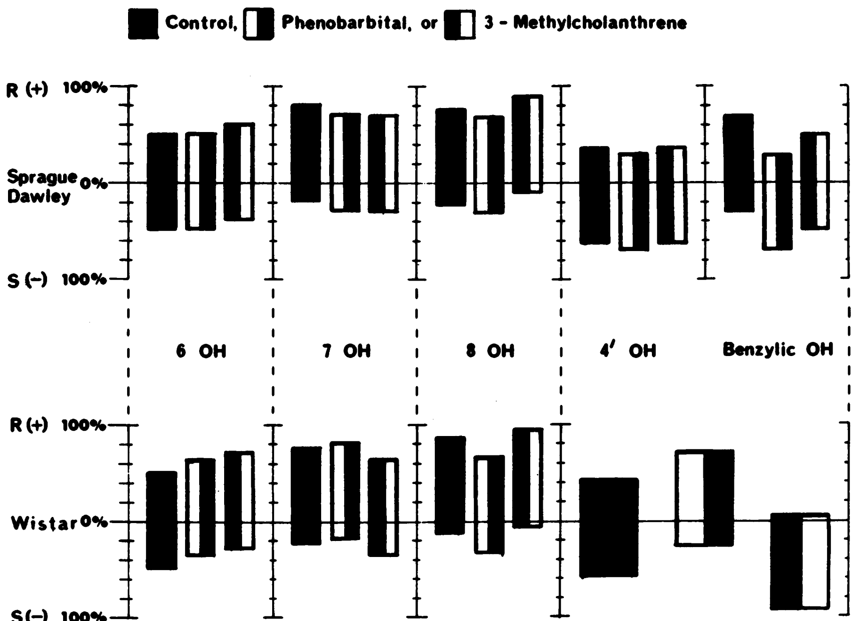

Figure 3. Stereoselectivity of the microsomal metabolism of the enantiomers of warfarin by normal-, PB-, and 3-MC-induced animals

(Figure 3). This behavior is in marked contrast to the similarity in stereoselectivity between the strains for 6- and 8-hydroxylation. These results clearly suggest that 7-hydroxylation is dissociated from 6-,8-hydroxylase activity and represents a distinct enzymatic process lending weight to our prior conclusions based on kinetic evidence.

The alterations in stereoselectivity following induction suggest that normal microsomes contain a 7-hydroxylase which is not inducible and which is distinct from the PB inducible 7-hydroxylase while changes in regioselectivity of hydroxylation of the coumarin ring caused by induction parallels those reported for simpler systems (32,33).

4'-Hydroxylation

The enzymatic activity for this process parallels that of the PB inducible 6- and 8-hydroxylase system but the reduced stereoselectivity suggests that non-PB inducible and non-3-MC inducible 4'-hydroxylase activity is present in normal microsomes.

Benzylic hydroxylation

The preferential formation of R-benzylic hydroxywarfarin at high substrate concentrations employing microsomes obtained from either rat strain was reported recently (59). In Sprague-Dawley rats 3-MC induction failed to increase the synthesis of this metabolite from either enantiomer whereas PB induction causes a greater increase in hydroxylation at this position than at any of the other aromatic positions except 7. Moreover, PB induction reverses the observed stereoselectivity, Figure 3, and a similar observation has been reported for another aliphatic hydroxylation (27). This finding demonstrates that normal microsomes contain a non-inducible benzylic hydroxylase which is stereoselective for the R-enantiomer and which is kinetically different from normal coumarin hydroxylase.

Differences in the stereochemical preferences obtained from the microsomal enzymes prepared from normal, PB or 3-MC pretreated animals permit several classes of hemoproteins to be distinguished. The simplist but by no means unique hypothesis consistent with our findings is that liver microsomes from normal animals contain at least four hemoprotein monoxygenases, enzymes A-D, differing in their stereoselectivity and regiospecificity toward the enantiomers of warfarin

These are classified as follows:

1) Enzyme A, which is present in normal animals but is not inducible by PB or 3-MC and is stereoselective and regioselective for 7- and benzylic hydroxylation of R-warfarin and 4'-hydroxylation of S-warfarin.

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2) Enzyme B, which is also present in normal animals, is inducible only by PB and is regioselective for 6-, 8- and some 4'-hydroxylation.

3) Enzyme C, which is present to a limited extent in normal animals, is inducible by PB only, and is regioselective for 7- and benzylic hydroxylation and more stereoselective for S-warfarin than Enzyme A.

4) Enzyme D, which is present in normal animals to a limited extent, is inducible only by 3-MC, and is stereoselective and regioselective for 6- and 8-hydroxylation of R-warfarin.

Perturbation of Product Ratios

As stated earlier the indirect method for studying multiplicity is not well defined and generally involves changes in the system induced by various treatments or perturbations. To our knowledge neither the assumptions nor the rules for their application have been explicitly stated by authors reporting such studies. Since a systematic framework for the interpretation of changes in enzymatic profiles induced by perturbations to the system would aid greatly in both the interpretation and the design of experiments, we are currently trying to develop such a framework utilizing the model of a single substrate and multiproducts (60). In developing the framework we assume that product formation is irreversible and that the steady state kinetics of Briggs and Haldane (61) are applicable.

For a single substrate, single enzymatic site, multiple product system, the following two cases are possible.

Case 1. The substrate combines with an enzyme to form a single enzyme-substrate complex which dissociates to multiple products. That is;

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \begin{cases} \xrightarrow{k_2} P_1 + E \\ \xrightarrow{k_3} P_2 + E \\ \xrightarrow{k_4} P_3 + E \end{cases}$$

Deriving the expression for E·S by the method of King and Altman (62) as described by Segel (63) and rearranging to the form of the Michaelis Menten equation yields

$$E{\cdot}S = \frac{(E_0)\ (S)}{\dfrac{k_{-1} + k_2 + k_3 + k_4}{k_1} + S}$$

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Therefore, a single Km = $\frac{k_{-1}+k_2+k_3+k_4}{k_1}$ characterizes the entire system and is independent of either enzyme or substrate concentration. Since the individual velocities for each of the products are $dP_1/dt = k_2(E \cdot S)$, $dP_2/dt = k_3(E \cdot S)$ and $dP_3/dt = k_4(E \cdot S)$ the ratio of any two will be constant and independent of either the concentration of E or S.

Case 2. The substrate combines with an enzyme to form energetically distinct E•S complexes each of which dissociates to a different product. That is;

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_4} P_1$$

$$\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E \cdot S' \xrightarrow{k_5} P_2$$

$$\underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E \cdot S'' \xrightarrow{k_6} P_3$$

Once again deriving the expressions for E•S, E•S' and E•S" by the method of King and Altman and rearranging in the form of the Michaelis-Menten equation yields three equations:

$$E \cdot S = \frac{\frac{k_1 (k_{-2}+k_5)(k_{-3}+k_6)[E_0][S]}{R}}{Km + S}$$

$$E \cdot S' = \frac{\frac{k_2 (k_{-1}+k_4)(k_3+k_6)[E_o][S]}{R}}{Km + S}$$

$$E \cdot S'' = \frac{\frac{k_3 (k_{-1}+k_4)(k_{-2}+k_5)[E_o][S]}{R}}{Km + S}$$

Where $R = k_1 (k_{-2}+k_5)(k_{-3}+k_6)+k_2(k_{-1}+k_4)(k_{-3}+k_6) + k_3(k_{-1}+k_4)(k_{-2}+k_5)$

$$\text{and } Km = \frac{(k_{-1}+k_4)(k_{-2}+k_5)(k_{-3}+k_6)}{R}$$

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The Km again characterizes the entire system. Since the velocities for product formation are $dP_1/dt = k_4(E{\cdot}S)$, $dP_2/dt = k_5(E{\cdot}S')$ and $dP_3/dt = k_6(E{\cdot}S'')$ and since the ratio of $(dP_1/dt)/dP_2dt) = \frac{k_4k_1(k_{-2}+k_5}{k_5k_2(k_{-1}+k_4)}$ the ratio of any two products is a constant which is independent of either enzyme or substrate concentration. It should be noted that in deriving the rate laws for Case 1 and Case 2 the only assumptions that were made were that steady state kinetics were applicable and that product formation was irreversible. It was not necessary to assume that product formation is rate limiting.

Consider a microsomal preparation which acts on a single substrate to yield multiple products. Information regarding the enzymatic multiplicity of the preparation can now be gained by perturbing the system. Fundamentally two types of perturbations are possible; 1) those that affect enzyme or substrate concentration without altering the active site or individual rate constants and 2) those that can alter the active site or individual rate constants.

Type-1 perturbers include such factors as inhibitors, inducers and substrate concentration studies. For such perturbations any statistically valid difference in the measured Km's or any change in product ratios for either Case 1 or Case 2 systems indicates the existence of at least two independent enzymatic sites. Conversely lack of changes in Km or product ratios are not conclusive evidence for the presence of a single enzymatic site but become increasingly convincing as the number of perturbations studied is increased.

Type-2 perturbers include such factors as pH (by affecting ionizable groups at the active site) ionic strength (change solvation of the active site) temperature (shift the steady state constants) and allosteric interactions (by inducing conformational changes at the active site). For such perturbations all combinations of changes in Km and product ratios are possible. That is, three results are possible: 1) Km and product ratios do not change. This result indicates that the perturbation did not affect either the rate constants or the active site; 2) Km changes but product ratios do not (e.g., a temperature effect) or product ratios change and Km does not (e.g., an allosteric interaction). These results indicate a single enzyme but are not conclusive (to have the situation where two independent enzymes gave exactly the same product ratios but had different Km's or the converse would be highly fortuitous); 3) Both Km and product ratios change. This result yields no information regarding multiplicity.

Based on the above analysis and assuming the model is applicable, it would appear that information regarding the multiplicity of microsomal cytochrome P-450's can most readily

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be obtained by perturbing the system by factors which only affect enzyme or substrate concentrations.

To test the method consider the warfarin data presented earlier in Table's I and II.

R-Warfarin

The statistical indistinguishability of the Km data for R-warfarin suggests that all the hydroxylated products could be formed by a single enzyme. If one accepts the arguments advanced for benzylic hydroxywarfarin having a different Km then at least two independent catalytic sites or enzymes are responsible.

The product ratios for each of the hydroxylated products from PB induced versus normal animals as determined from Table II are shown below:

$$\text{6-OH}\ \frac{PB}{Normal} = 1.96 \pm .15 \qquad \text{7-OH}\ \frac{PB}{Normal} = 2.27 \pm .20$$

$$\text{8-OH}\ \frac{PB}{Normal} = 2.13 \pm .14 \qquad \text{4'-OH}\ \frac{PB}{Normal} = 2.34 \pm .15$$

$$\text{benzylic-OH}\ \frac{PB}{Normal} = 1.5 \pm .18$$

The aromatic hydroxylation processes are statistically indistinguishable while at the 90-95% confidence level benzylic hydroxylation is differentially induced.

S-Warfarin

The PB/Normal product ratios for S-warfarin are shown below:

$$\text{6-OH}\ \frac{PB}{Normal} = 1.92 \pm 0.12 \qquad \text{7-OH}\ \frac{PB}{Normal} = 3.95 \pm .15$$

$$\text{8-OH}\ \frac{PB}{Normal} = 3.23 \pm .29 \qquad \text{4'-OH}\ \frac{PB}{Normal} = 1.75 \pm 0.22$$

$$\text{benzylic-OH}\ \frac{PB}{Normal} = 8.54 \pm 0.86$$

In contrast to R-warfarin three product groupings are immediately apparent. These are 6- and 4'-hydroxylation, 7- and 8-hydroxylation and benzylic hydroxylation. Based on the difference in Km a further distinction between 7- and 8-hydroxylation can be made. Thus, S-warfarin is hydroxylated by at least four distinct enzymatic processes.

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R + S-Warfarin

Thus far the analysis has considered a single substrate only. To compare two different substrates one must establish that they are interacting with the same enzymatic systems. Since biological systems are chiral, enantiomers must be considered as different substrates. In the analysis to follow we assume that the R and S enantiomers are interacting with the same group of enzymes. Experiments are in progress to clarify this assumption by utilizing one enantiomer as a competitive inhibitor for the other.

Assume for example, that a single enzyme generates both 6-(R) and 6-(S)-hydroxywarfarin then the ratio of 6-OH $\frac{R}{S}$ should be constant upon perturbation of enzyme or substrate concentration. Therefore

$$6\text{-OH}\frac{R}{S}\ \text{Normal} = 6\text{-OH}\frac{R}{S}\ \text{PB}$$

which implies that $R\text{-6-OH}\ \frac{PB}{Normal} = S\text{-6-OH}\ \frac{PB}{Normal}$

This relationship holds for the comparison of any two products and therefore all the necessary calculations have already been done. Groups of statistically non-distinguishable products obtained from the product ratios calculated above are shown below.

Group 1

R-6-OH = 1.96±.15
R-7-OH = 2.27±.20
R-8-OH = 2.13±.14
R-4'-OH = 2.34±.15
S-6-OH = 1.92±.12
S-4'-OH = 1.75±0.22

Group 2

R-benzylic-OH 1.5±.18

Group 3

S-benzylic-OH 8.54±.86

Group 4

S-7-OH = 3.95±.15

Group 5 (Based on Km)

S-8-OH = 3.23±.29

Thus the analysis indicates that the combination of normal and PB induced microsomes contain a minimum of five distinct enzymatic processes. Further differentiation and determination of the individual catalytic regio and stereospecificities requires the study of a greater number of perturbations and ultimately the study of the isolated hemoproteins themselves. Such studies are currently in progress. For example a similar analysis of the 3-MC data indicates that this agent induces the formation of apparently abnormal isozymes which catalyze 6- and 8-hydroxylation. The isozymes responsible for the remaining hydroxylation reactions are not induced.

The application of the product ratio technique to the interpretation of microsomal data appears to be reasonably successful and if valid, greatly simplifies such interpretations. The applicability of this method to cases where Michaelis Menton

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kinetics are not followed such as: product inhibition, substrate inhibition, non hyperbolic kinetics in general; to different mechanisms for P-450 oxidation; to the influence of epoxide hydrase etc., are being studied.

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6

Role of Purified Cytochrome P-448 and Epoxide Hydrase in the Activation and Detoxification of Benzo[*a*]pyrene

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The liver microsomal monoxygenase system is a membrane-bound, multicomponent electron transport system which is responsible for the oxidative metabolism of a variety of endogenous and exogenous substrates such as steroids, fatty acids, drugs, insecticides and chemical carcinogens (1). Of the three components involved in microsomal drug metabolism (cytochrome P-450, NADPH-cytochrome c reductase and phosphatidylcholine), cytochrome P-450 is undoubtedly the most important because of its vital role in oxygen activation, substrate binding and in determining the overall substrate specificity of the enzyme system (2,3). The rate at which various compounds are metabolized by this enzyme system varies widely and depends on the species, strain, age, tissue and pretreatment of the animal (1). Over the last decade, numerous studies have suggested that different forms of cytochrome P-450 exist in liver microsomes, and, more recently, the purification and reconstitution of the monoxygenase system have established the existance of multiple forms of cytochrome P-450 having different substrate specificities. The various purified forms of cytochrome P-450 differ from one another not only in their substrate specificity but also in their spectral and immunological properties as well as in their minimum molecular weights as determined by SDS-gel electrophoresis (4-9).

The importance of this rather versatile enzyme system has become increasingly apparent during the last 10 years. Today we live in a society that has become increasingly aware of the potential dangers of environmental pollutants which include chemical carcinogens. It has been estimated that 60-80% of all human cancers are caused by environmental factors (10-12), and many chemicals in our environment are metabolically activated to ultimate carcinogens by the microsomal monoxygenase system (13). One

of these environmental pollutants, the polycyclic aromatic hydrocarbon benzo[a]pyrene (BP),[1] may be one of the most prevalent chemical carcinogens to which man is exposed (14).

Since polycyclic hydrocarbons such as BP are chemically inert, their carcinogenicity is thought to result from metabolic activation by the microsomal monoxygenase system to a chemically reactive intermediate(s)(13,15-17). The oxidative metabolism of BP proceeds initially through the formation of reactive arene oxides which spontaneously isomerize to phenols, are hydrated to dihydrodiols by microsomal epoxide hydrase or are conjugated with glutathione via the soluble glutathione S-transferases (15, 16,18). In order to elucidate the role of metabolism in the mutagenicity and carcinogenicity of BP, a basic understanding of the properties and mechanism of action of the enzymes involved in the activation and inactivation of BP is essential. Thus, the purification and reconstitution of the monoxygenase system (19) in the presence or absence of purified epoxide hydrase (20) has enabled us to study the role of these enzymes in the metabolism of BP and BP derivatives and to manipulate the source of the purified cytochrome P-450 as well as the level of epoxide hydrase to generate mutagenic metabolites of this polycyclic aromatic hydrocarbon. Finally, the synthesis of approximately thirty BP derivatives and metabolites (21) has permitted us to utilize these compounds as substrates for the purified monoxygenase system in an effort to identify the bioactivated metabolites of BP.

Metabolism of Benzo[a]pyrene and Benzo[a]pyrene Arene Oxides by the Purified Monoxygenase System and Epoxide Hydrase. The purified, reconstituted monoxygenase system with and without addition of purified epoxide hydrase has been utilized to study the metabolism of [^{14}C]-benzo[a]pyrene (Table I). Although high pressure liquid chromatography is highly efficient for the separation and quantitation of dihydrodiols, phenols and quinones formed from BP, all potential metabolites within each group have not been identified in these studies (22). Thus, diol fractions 1, 2 and 3 correspond to BP 9,10-, 4,5- and 7,8-dihydrodiols, quinone fraction 1 corresponds to BP 1,6-, 3,6- and 4,5-quinones, quinone fraction 2 corresponds to BP 11,12- and 6,12-quinones and BP 4,5-oxide, phenol fraction 1 corresponds to 2-, 6-, 8- and 9-HOBP and phenol fraction 2 corresponds to the other 8 isomeric phenols of BP (1-, 3-, 4-, 5-, 7-, 10-, 11- and 12-HOBP). In the absence of epoxide hydrase, the reconstituted cytochrome P-448 containing monoxygenase system metabolizes BP to phenols and quinones (Table I). Upon addition of purified epoxide hydrase, the rate of total BP metabolism is unchanged, but significant amounts of dihydrodiols are produced at the expense of phenols. Formation of diol fractions 2 and 3 (BP 4,5- and 7,8-dihydrodiols, respectively) reached a maximum level with the addition of 5 units of epoxide hydrase whereas further addition

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of the enzyme resulted in a continued increase in diol fraction 1 (BP 9,10-dihydrodiol), probably as a result of the marked instability of BP 9,10-oxide ($t_{1/2} \leq 2$ minutes at 37° in 100 mM potassium phosphate buffer) compared to BP 4,5- and 7,8-oxides. Thus, higher amounts of epoxide hydrase would be required to

Table I

Metabolism of Benzo[a]pyrene by a Purified Cytochrome P-448 Dependent Monoxygenase System and Epoxide Hydrase

Epoxide Hydrase	Diol 1	Diol 2	Diol 3	Quinone 1	Quinone 2	Phenol 1	Phenol 2	Total
(units)	(nmol product formed/nmol hemeprotein/min)							
None	0.06	0.05	0.07	1.54	0.52	0.87	1.31	4.42
5	0.59	0.41	0.57	1.66	-	0.63	1.00	4.86
15	0.79	0.47	0.61	1.62	-	0.35	0.90	4.74
50	1.19	0.48	0.56	1.39	-	0.13	0.74	4.49

Incubation mixtures contained 0.2 nmol cytochrome P-448, 120 units of NADPH cytochrome c reductase, 0.1 mg of lipid, 0.5 µmol of NADPH, 3 µmol of $MgCl_2$, 100 µmol of potassium phosphate buffer (pH 6.8) and 95 nmol of [^{14}C]-BP in a final volume of 1 ml. One unit of epoxide hydrase produces 1 nmol of styrene glycol per min from styrene oxide. Metabolites of BP were analyzed by high pressure liquid chromatography as described by Holder et al. (22).

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completely convert BP 9,10-oxide to the corresponding dihydrodiol. A good stoichiometric relationship was found between the increase in the diol 2 fraction and the decrease in quinone fraction 2 which contains mainly BP 4,5-oxide. Direct confirmation that arene oxides of BP are substrates for the purified epoxide hydrase is shown in Table II. BP 4,5-, 7,8- and 9,10-oxides are all metabolized to the corresponding dihydrodiols at comparable rates using the purified enzyme while BP 11,12-oxide is a relatively poor substrate for the enzyme. No detectable BP 11,12-dihydrodiol is formed by the purified cytochrome P-448 containing monoxygenase system in the presence of epoxide hydrase. The results of our studies on the metabolism of BP in the presence or absence of epoxide hydrase demonstrate that the dihydrodiols and phenolic metabolites of BP share arene oxides as common precursors.

Requirements for the Metabolic Activation of Benzo[a]pyrene to Mutagenic Products by a Purified Monoxygenase System. Mutagenicity tests utilizing microorganisms or cultured mammalian cells have been used with increasing frequency to identify bioactivated metabolites of carcinogens. Metabolic activation of chemicals to mutagenic metabolites has been most commonly performed by a procedure developed by Ames and his associates (23, 24). Generally, the chemical, bacteria and appropriate cofactors are incubated with microsomes or a 9000 xg supernatant fraction of liver in a semisolid agar gel for 48 hours. When little is known about the metabolism of a particular compound, or a large number of diverse chemicals are being evaluated for mutagenic activity, relatively crude tissue homogenates should be used as the source of enzymes to ensure that all possible metabolic pathways are being evaluated. While this procedure has been used successfully to activate a number of carcinogens to bacterial mutagens, it has limitations for identifying the mutagenic metabolites formed from a compound which undergoes oxidation via multiple pathways. The 9000 xg supernatant fraction is relatively crude and contains many enzymatic and structural proteins, nucleic acids and numerous nucleophilic and electrophilic groups which could interact with the bioactivated metabolites before they reach the bacteria. Moreover, regardless of the source of the monoxygenase activity, long incubation times can result in spontaneous or enzymatic breakdown of primary metabolites to as yet ill-defined products (25,26). An examination of the metabolite profile obtained from BP when liver microsomes are incubated for 30 minutes with limiting substrate concentrations revealed that all primary oxidative products of BP (dihydrodiols, quinones and phenols) undergo extensive secondary metabolism by the monoxygenase system (26). Thus, use of prolonged incubation times for metabolic activation studies in agar gel would all but eliminate the possibility of obtaining a profile of the metabolites formed under the conditions which induce mu-

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Table II

Metabolism of Benzo(a)pyrene Arene Oxides by Purified Epoxide Hydrase

Substrate	Dihydrodiol Formed (nmol/mg protein/min)
BP 4,5-oxide	445
BP 7,8-oxide	321
BP 9,10-oxide	390
BP 11,12-oxide	31

Incubation mixtures contained 2-6 μg of purified epoxide hydrase, 30 μg of phosphatidylcholine, 12.5 μmol of Tris buffer and 10-25 nmol of tritium labelled substrate in a final volume of 80 μl.

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tations. Finally, the ability to manipulate the amount of various BP metabolites formed through alteration of the ratio of epoxide hydrase to the monoxygenase system (cf Table I) should be of value in determining the nature of the bioactivated metabolites formed from BP. We, therefore, sought to develop an enzymatically well-defined monoxygenase system which would metabolize BP and BP derivatives to mutagenic products under conditions which would permit the analysis and identification of the metabolites (27).

Except for the presence of bacteria (*Salmonella typhimurium* strain TA 98) in the reaction mixture, the metabolism of BP to mutagenic products by the reconstituted system was performed essentially as described for metabolite identification. Bacteria (2×10^8 cells) were suspended in a total incubation volume of 0.5 ml containing 2.5 μmol of sodium phosphate, 75 μmol of sodium chloride, 0.08 μmol (50 μg) of phosphatidylcholine, 150 units of NADPH-cytochrome c reductase, 0.02-0.2 nmol of cytochrome P-450 or P-448, 25 nmol of BP (in 12.5 μl acetone) and 0.1 μmol of NADPH. The final pH of the incubation mixture was 6.8. After incubation at 37° for 5 minutes, 9 nmol of menadione was added to stop the reaction followed immediately by 2.0 ml of molten top agar, and the standard Ames pour plate procedure (24) was performed. Studies on the requirements for the enzymatic activation of BP by the reconstituted cytochrome P-448 system indicated that optimal metabolic activation to mutagenic metabolites required the presence of NADPH, NADPH-cytochrome c reductase, cytochrome P-448 and phosphatidylcholine (Table III). A 5 minute incubation with 0.1 nmol of cytochrome P-448 and saturating amounts of NADPH-cytochrome c reductase, phospholipid, NADPH and BP induced approximately a 15-fold increase in histidine-independent colonies in strain TA 98. An absolute requirement was observed for all components of the system except phosphatidylcholine which is in agreement with the required components for the metabolism of BP to phenolic metabolites (19). In other experiments (27), it was established that the number of mutations induced in *Salmonella typhimurium* strain TA 98 was proportional to the amount of added cytochrome P-448 and to the time of incubation (Figure 1).

Metabolism of Benzo(a)pyrene to Mutagenic Metabolites by Various Purified Forms of Cytochrome P-450. In the last decade, numerous laboratories have investigated the possibility that multiple hydroxylase systems exist in liver microsomes. The purification and reconstitution of this enzyme system has provided the means to study in detail the physical properties of each of the components and the catalytic activity of cytochrome P-450. These studies have provided direct evidence for the existance of multiple forms of cytochrome P-450, each of which have different, but overlapping, substrate specificities (4-7). Table IV shows a comparison of the metabolism of BP to mutagenic

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Table III

Requirements for the Metabolic Activation of Benzo[a]pyrene to Mutagenic Products in Salmonella Typhimurium Strain TA 98

Addition	His^+ Revertants/Plate	Benzo[a]pyrene Metabolism (% Activity)
None	36	-
BP	37	-
Complete Monoxygenase System	440	100
-BP	30	0
-NADPH-cytochrome c reductase	32	1
-Cytochrome P-448	32	2
-Phosphatidylcholine	74	18
-NADPH	34	0

The complete monoxygenase system consisted of 50 μg of phosphatidycholine, 150 units of NADPH-cytochrome c reductase, 0.1 nmol of cytochrome P-448, 0.1 μmol of NADPH and 25 nmol of BP in a final volume of 0.5 ml containing 2 x 10^8 bacteria. Incubations were at 37° for 5 min.

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Table IV

Metabolism of Benzo(a)pyrene to Mutagenic Products by Various Purified Forms of Cytochrome P-450

Source of Cytochrome P-450		Benzo(a)pyrene Metabolism (pmol formed/ pmol hemeprotein)	Mutations in Strain TA 98 (His+ Revertants/pmol hemeprotein)
Species	Pretreatment of Animals		
Rat	3-Methylcholanthrene	15.0	38.0
Rat	Aroclor 1254	13.0	26.5
Rat	Phenobarbital	0.7	0.55
Rabbit	3-Methylcholanthrene	0.7	0.25
Mouse	Phenobarbital, Fraction A_2	0.5	0.05
Mouse	Phenobarbital, Fraction C_2	0.1	0.05

All incubation mixtures contained the complete monoxygenase system and 2 x 10^8 bacteria in a final volume of 0.5 ml as described in Table III. Cytochrome P-450 was purified from the livers of rats (5,29), rabbits (6) or mice (7) as previously described. The final substrate concentration was 25 µM. After incubation for 5 min at 37°C, the reactions were terminated by the addition of 9 nmol of menadione. Benzo(a)pyrene hydroxylation was measured as fluorescent phenols (28).

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metabolites by the purified and reconstituted monoxygenase system using cytochrome P-450 purified from animals treated with different inducers as well as from several different animal species. These results clearly demonstrate the marked differences in the metabolism of BP to fluorescent phenols by various purified forms of cytochrome P-450. The metabolic activation of BP to products mutagenic to strain TA 98 of S. typhimurium by various purified cytochrome P-450's was similar to the rate of metabolism of BP to fluorescent phenols (Table IV). Cytochrome P-450 isolated from rats pretreated with either 3-methylcholanthrene or Aroclor 1254 were the most efficient hemeproteins for the metabolism of BP to fluorescent phenols and mutagenic products while the other purified cytochrome P-450's were much less effective.

Effect of Epoxide Hydrase on the Metabolic Activation of Benzo[a]pyrene to Mutagenic Metabolites. Epoxide hydrase is an important enzyme in the metabolism of BP since it converts the intermediate arene oxides formed by the monoxygenase system to the corresponding trans dihydrodiols (15,16). In the absence of further metabolic activation, the dihydrodiols produced from these arene oxides are essentially nontoxic (21,30-32). Addition of highly purified epoxide hydrase to the monoxygenase system during the metabolism of BP decreased the mutation frequency by a maximum of 30% in S. typhimurium strain TA 98 (Figure 2), indicating that at least some of the mutagenic metabolites of BP are arene oxides. In contrast to these results, mutations induced by BP 4,5-oxide were completely abolished by the addition of 5 units of epoxide hydrase (Figure 2). Thus, the inability of epoxide hydrase to completely abolish the metabolic activation of BP to mutagenic products suggested that non-arene oxide metabolites of BP may also have mutagenic activity or that some mutagenic epoxide metabolites are poor substrates for epoxide hydrase.

Metabolic Activation of Benzo[a]pyrene Phenols. Phenols, formed either by spontaneous isomerization of arene oxides (15) or direct oxygen insertion reactions (33), are the primary oxidative products when BP is metabolized by the purified monoxygenase system in the absence of epoxide hydrase (cf Table I). Of the twelve possible isomeric phenols of BP, only 6- and 12-HOBP have significant intrinsic mutagenic activity in several strains of S. typhimurium (32). However, based on the amount of phenols produced from BP by the purified monoxygenase system (cf Table I), it is unlikely that these phenols contribute significantly to the mutagenicity observed from BP metabolism. Since several studies have shown that primary oxidative metabolites of BP, including phenols, can be further metabolized by the monoxygenase system (26,34), we utilized all twelve of the phenols as substrates for the purified monoxygenase system to determine if further oxidative metabolism would result in the formation of mutagenic products. Table V shows the mutagenic

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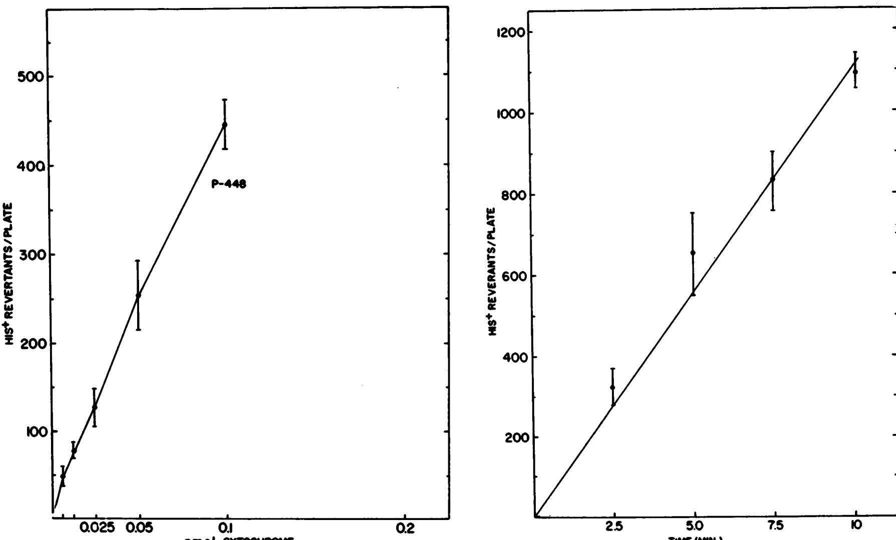

Figure 1. Effect of cytochrome P-448 concentration and time of incubation on the metabolism of BP to products mutagenic to strain TA 98 of Salmonella typhimurium. *Reaction mixtures were similar to those described in Table III. Each value represents the mean ± S.D. from three replicate incubation mixtures.*

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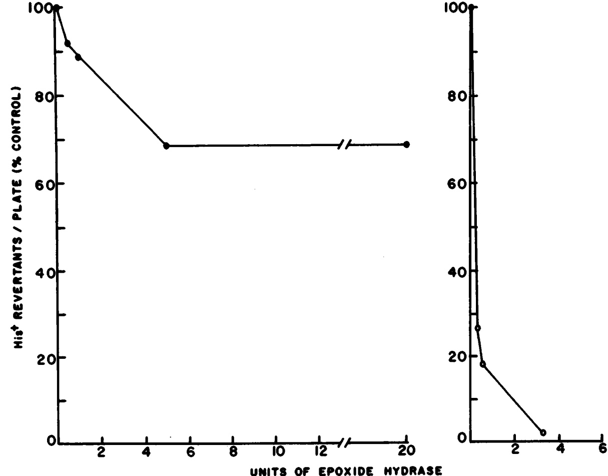

Figure 2. Effect of epoxide hydrase on mutations induced by the metabolism of BP by the purified monoxygenase system (left) and on the mutagenic activity of BP 4,5-oxide (right).

Incubation mixtures for the metabolic activation of BP were similar to those described in Table III. The effect of epoxide hydrase on the mutagenic activity of BP 4,5-oxide was assayed using 0.4 nmole of BP 4,5-oxide in 0.5 ml containing 2 × 10^8 bacteria. All samples were incubated for 5 min at 37° before addition of the top agar.

Table V

Metabolism of Benzo(a)pyrene Phenols to Mutagenic Products by a Purified Cytochrome P-448 Dependent Monoxygenase System

Substrate	Cytochrome P-448 (pmol)		
	0	25	50
	His$^+$ Revertants/Plate		
None	17	-	17
BP	18	280	670
1-HOBP	43	109	140
2-HOBP	17	67	73
3-HOBP	32	117	158
4-HOBP	15	22	29
5-HOBP	19	19	24
6-HOBP	94	116	261
7-HOBP	35	43	49
8-HOBP	15	35	45
9-HOBP	33	93	180
10-HOBP	14	22	21
11-HOBP	11	21	21
12-HOBP	219	302	372

Incubation mixtures contained the complete monoxygenase system and *S. typhimurium* strain TA 98 as described in Table III using fixed concentrations of all components except cytochrome P-448. The final concentration of BP or BP phenols was 25 μM.

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activity of the twelve isomeric phenols of BP in strain TA 98 before and after metabolic activation by various amounts of cytochrome P-448. The number of revertant colonies in the absence of the cytochrome is a measure of the intrinsic mutagenic activity of the phenols since the reconstituted system is inactive in the absence of the cytochrome. Of the twelve possible phenols of BP, only 1-, 2-, 3-, 6-, 9- and 12-HOBP were metabolically activated to mutagenic products. However, since none of the phenols was as effectively converted to mutagenic products as BP, it appears that further metabolism of BP phenols to mutagenic products is not responsible for the mutagenic activity of metabolically activated BP. This data, together with the lack of inherent mutagenic activity of six possible BP quinones (32), strongly suggests that nonepoxide derivatives of BP are not responsible for the mutations observed when BP is metabolized to mutagenic products in the presence of the purified monoxygenase system and epoxide hydrase.

Metabolic Activation of Benzo[a]pyrene Dihydrodiols. Since addition of epoxide hydrase to the purified monoxygenase system was unable to block the formation of mutagenic metabolites of BP, it was possible that an arene oxide of BP was converted to a dihydrodiol by addition of epoxide hydrase which was then further metabolically activated to a potent mutagen. In this regard, Borgen *et al.* (35) have shown that metabolic activation of BP 7,8-dihydrodiol by liver microsomes results in a much greater binding to DNA than does such metabolic activation of BP, BP 4,5-dihydrodiol or BP 9,10-dihydrodiol. Sims *et al.*(36) have presented evidence that a BP 7,8-diol-9,10-epoxide is the bioactivated metabolite of BP 7,8-dihydrodiol which binds to DNA. It has since been established that the stereoisomeric BP 7,8-diol-9,10-epoxides are among the most potent mutagens yet described (21,31,37-39). We have utilized the purified monoxygenase system in an attempt to metabolically activate the four BP dihydrodiols which have been synthesized in our laboratories (21). As shown in Figure 3, BP 7,8-dihydrodiol was activated to at least a 4-fold greater extent than was BP. In contrast to these results, the BP 4,5-, 9,10- and 11,12-dihydrodiols were not metabolically activated to mutagenic metabolites. The high mutagenic activity of a metabolite(s) of BP 7,8-dihydrodiol towards *S. typhimurium* strain TA 98 prompted further studies on the metabolic activation of BP 7,8-dihydrodiol by the purified monoxygenase system. Microsomal epoxide hydrase converts the arene oxides formed by the cytochrome P-450 monoxygenase system into *trans*, vicinal dihydrodiols (15). Table VI shows that *cis* BP 7,8-dihydrodiol can also be metabolically activated to a potent mutagen(s) by the monoxygenase system, although it is slightly less active than the *trans* isomer. These results indicate that the relative position of the hydroxyl groups is not a critical factor in determining the mutagenic activity of the

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Table VI

Metabolic Activation of Benzo[a]pyrene 7,8-Dihydrodiol to Mutagenic Products

Substrate	Cytochrome P-448 (pmol)					
	0	2	5	10	20	80
	His$^+$ Revertants/Plate					
BP	1±0.6	9±2	34±3	60±5	120±10	800±45
(±)-*trans* BP 7,8-dihydrodiol	6±1	130±8	522±45	-	-	-
(±)-*cis* BP 7,8-dihydrodiol	1±0.5	76±10	320±18	-	-	-
(±)-*trans* BP 7,8-dihydroxy-7,8,9,10-H_4BP	2±1	-	6±4	10±3	9±2	29±4

Incubation mixtures contained the complete monoxygenase system as described in Table III. The final substrate concentration was 25 µM. Background mutation frequencies have been subtracted. Values represent the mean ± S.E. for three determinations.

bioactivated metabolite(s). However, 7,8-dihydroxy-7,8,9,10-tetrahydro BP, a compound related to BP 7,8-dihydrodiol but with the double bond removed from the 9,10-position of the molecule, cannot be metabolically activated to mutagenic metabolites by the monoxygenase system. These results suggest that the mutagenic product formed on metabolic conversion of BP 7,8-dihydrodiol is a BP 7,8-diol-9,10-epoxide since the double bond in the 9,10-position of the molecule is a requirement for the formation of an epoxide at that position.

Metabolic Activation of Benzo[a]pyrene 7,8-Oxide. Since BP 7,8-dihydrodiol was readily metabolized to a mutagenic product(s) by the cytochrome P-448 monoxygenase system, we examined the effect of the monoxygenase system on the mutagenic activity of its arene oxide precursor, BP 7,8-oxide (Table VII). In the absence of added epoxide hydrase, the weak intrinsic mutagenic activity of BP 7,8-oxide (40) was unaffected by addition of the purified monoxygenase system. Addition of epoxide hydrase to the monoxygenase system resulted in a marked increase in the mutation frequency in S. typhimurium strain TA 98. These results demonstrate that BP 7,8-oxide is hydrated to the corresponding dihydrodiol which in turn is oxidatively metabolized to an active mutagen(s), presumably the stereoisomeric BP 7,8-diol-9,10-epoxides.

Metabolism of Benzo[a]pyrene 7,8-Dihydrodiol by the Purified Monoxygenase System. Based on the studies of Borgen et al.(35), Sims et al.(36) proposed a BP 7,8-diol-9,10-epoxide as the bioactivated metabolite of BP 7,8-dihydrodiol which binds to DNA. The oxidative metabolism of BP 7,8-dihydrodiol at the 9,10-position of the molecule could result in the formation of two possible stereoisomers of the diol epoxide (Figure 4). Recently our laboratories (41) synthesized and unequivocally assigned the relative stereochemistry of the two stereoisomers of BP 7,8-diol-9,10-epoxide (diol epoxides 1 and 2), both of which are potent mutagens (31,37-39). A detailed study of the metabolism of BP-7,8-dihydrodiol is thus necessary in order to determine whether one or both of the stereoisomers are responsible for the mutagenic activity of metabolically activated BP 7,8-dihydrodiol. Recently, high pressure liquid chromatography has been used to separate metabolites of BP 7,8-dihydrodiol (42,43). Although a direct demonstration of the formation of the diol epoxides has not been possible due to their extreme instability in aqueous solutions, tetraols have been identified as metabolites from BP 7,8-dihydrodiol (Figure 4). Diol epoxides 1 and 2 undergo cis and trans addition of water at the 10-position of the oxirane (42,43) to form stereoisomeric pairs of (±)-7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydro BP derivatives in which the relative stereochemistry of the 7- and 8-hydroxyl group is always trans and the relative stereochemistry between the 8- and 9-

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Table VII

Metabolic Activation of BP 7,8-oxide to Mutagenic Metabolites

Substrate	Monoxygenase System	Epoxide Hydrase	His^+ Revertants/Plate
BP 7,8-oxide (25 μM)	-	-	91±4
	+	-	132±10
	+	+	514±22

The complete monoxygenase system consisted of 0.05 nmol of cytochrome P-448, 150 units of NADPH-cytochrome c reductase, 50 μg of phosphatidylcholine, 0.1 μmol of NADPH and 2 x 10^8 bacteria in a final volume of 0.5 ml. Four units of purified epoxide hydrase was added to the appropriate reaction mixtures (see Table I for definition of units of epoxide hydrase activity). The mutations observed in the absence of the monoxygenase system and epoxide hydrase are a result of the inherent mutagenicity of BP 7,8-oxide. Background mutation frequencies have been subtracted. Values repesent the mean ± S.E. for three determinations.

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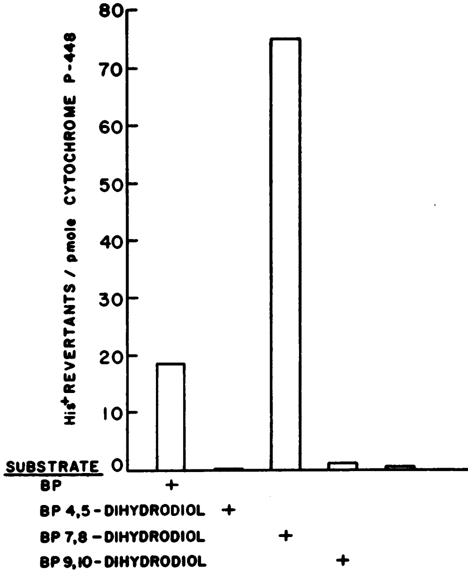

Figure 3. Metabolic activation of (±)-trans BP dihydrodiols. Samples were incubated with the purified monoxygenase system as described in Table III using 25 μM substrate concentration and 0.075–0.10 nmol cytochrome P-448.

BP 7,8-Dihydrodiol

Diol Epoxide 1

Diol Epoxide 2

cis 1

trans 1

cis 2

trans 2

Figure 4. Metabolism of BP 7,8-dihydrodiol to the stereoisomeric BP 7,8-diol-9,10-epoxides. Tetraols are formed upon cis and trans addition of water at the 10-position.

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hydroxyl groups is determined by the starting diol epoxide (Figure 4). When authentic diol epoxides 1 and 2 are incubated in potassium phosphate buffer (pH 7.4) at 37°, diol epoxide 1 forms 85% cis 1 and 15% trans 1 while diol epoxide 2 forms 7% cis 2 and 93% trans 2 by respective cis and trans addition of water (43). Diol epoxides incubated in the presence of enzymes give slightly different ratios, probably as a result of the hydrophobicity of the enzyme system.

Metabolism of (±)-trans-BP 7,8-dihydrodiol was studied using the purified monoxygenase system containing either rat liver cytochrome P-450 or P-448 (Table VIII). When the system was reconstituted with cytochrome P-448, diol epoxide 2 was the major metabolite, accounting for as much as 55% of the total metabolism while diol epoxide 1 accounted for 35-40% of the total metabolism. Other unknown metabolites are formed which include a phenolic derivative (43,44) and triols which are formed via nonenzymatic reduction of tetraols by NADPH (42). Cytochrome P-450 from phenobarbital-treated rats is markedly less effective in metabolizing BP 7,8-dihydrodiol, as is also the case when BP is the substrate for the enzymes. The addition of epoxide hydrase to the purified monoxygenase system has no effect on the profile of metabolites formed from BP 7,8-dihydrodiol, indicating that the diol epoxides of BP are not substrates for this enzyme. Mutagenesis studies (27,39) have also shown that the stereoisomeric BP 7,8-diol-9,10-epoxides are poor substrates for epoxide hydrase (Figure 5). The lack of effect of epoxide hydrase on the diol epoxides is probably not a result of their extreme instability (39), since H_4-9,10-epoxide is metabolically inactivated by the enzyme and this compound has a half-life in buffer which is similar (39) to that for diol epoxide 2 (Figure 5). Thus, the enzyme epoxide hydrase, long considered to be a detoxifying enzyme, can play a variety of roles in the metabolic inactivation and activation of BP to mutagenic metabolites: (1) BP 4,5-oxide is a substrate for epoxide hydrase and is inactivated by this enzyme. (2) BP 7,8-oxide is metabolically activated to a mutagenic metabolite(s) in the presence of both epoxide hydrase and the monoxygenase system and (3) the stereoisomeric BP 7,8-diol-9,10-epoxides are not substrates for the enzyme.

Since several forms of highly purified cytochrome P-450 (P-448) have been shown to metabolize BP at significantly different rates, it was of interest to determine the ability of the various purified cytochromes to metabolize (±)-trans-BP 7,8-dihydrodiol to mutagenic metabolites. Table IX shows that the cytochrome P-450's isolated from 3-methylcholanthrene or Aroclor 1254-pretreated rats were the most effective hemeproteins for the metabolism of BP 7,8-dihydrodiol to mutagenic products. The various other forms of purified cytochrome P-450 metabolized BP 7,8-dihydrodiol to mutagenic metabolites but at a much lower rate. Interestingly, the results obtained with BP 7,8-dihydrodiol as substrate parallel the results obtained for these heme-

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Table VIII

Metabolism of (±)-trans-Benzo[a]pyrene 7,8-Dihydrodiol by a Reconstituted Monoxygenase System

Cytochrome	Epoxide Hydrase	Metabolites Trans 1	Cis 1	Trans 2	Cis 2	Unknown	Total
		(nmol formed/nmol hemeprotein/minute)					
P-448	-	0.14	0.61	1.03	0.08	0.18	2.69
P-448	+	0.17	0.60	1.00	0.08	0.28	2.77
P-450	-	0.005	0.02	0.06	0.004	0.005	0.10
P-450	+	0.004	0.03	0.06	0.06	0.000	0.10

Incubation mixtures contained 0.2 nmol of cytochrome P-448 or 0.4 nmol of cytochrome P-450, 225-450 units of NADPH-cytochrome c reductase, 30-50 µg of phosphatidylcholine, 34 units of epoxide hydrase, 0.5 µmol of NADPH, 3 µmol of $MgCl_2$, 100 µmol of potassium phosphate buffer (pH 6.8) and 40 nmol of [^3H]-(±)-trans-BP 7,8-dihydrodiol in a final volume of 1 ml. Samples were incubated for 10 minutes at 37°.

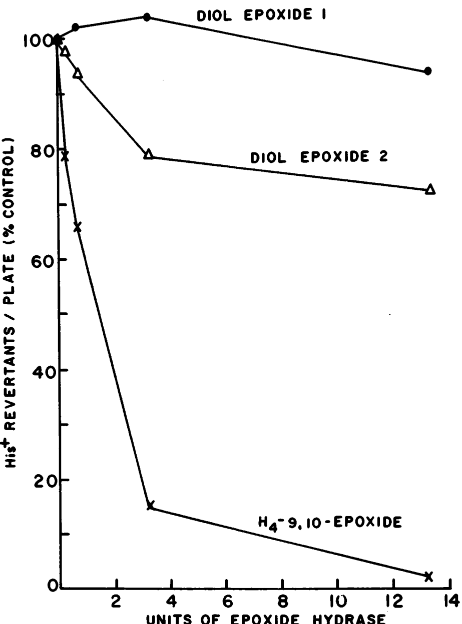

Figure 5. Effect of epoxide hydrase on the mutagenic activity of the stereoisomeric BP 7,8-diol-9,10-epoxides (diol epoxide 1 and 2) and H_4-9,10-epoxide.

Incubation mixtures consisted of 0.5 ml containing diol epoxide 1 (0.1 nmol), diol epoxide 2 (0.1 nmol) or H_4-9,10-epoxide (0.05 nmol), 2 × 10^8 bacteria, and the indicated amount of purified epoxide hydrase. After a 5-min incubation at 37°, top agar (2.0 ml) was added, and the plates were poured. The number of revertants induced per nmol of each derivative in the absence of epoxide hydrase was: diol epoxide 1, 3780; diol epoxide 2, 2650; H_4-9,10-epoxide, 6100.

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Table IX

Metabolism of (±)-trans-Benzo[a]pyrene 7,8-Dihydrodiol to Mutagenic Products by Various Purified Forms of Cytochrome P-450

Source of Cytochrome P-450		His^+ Revertants/
Species	Pretreatment of Animals	pmol Hemeprotein
Rat	3-Methylcholanthrene	435
Rat	Aroclor 1254	320
Rat	Phenobarbital	17
Rabbit	3-Methylcholanthrene	19
Mouse	Phenobarbital, Fraction A_2	2
Mouse	Phenobarbital, Fraction C_2	5

All incubation mixtures contained 50 g of phosphatidylcholine, 150 units of NADPH-cyrochrome c reductase, 1.5-15 pmol of purified cytochrome P-450, 2 x 10^8 bacteria and 25 μM substrate in a final volume of 0.5 ml. Incubations were at 37° for 5 min. Background mutation frequencies were subtracted.

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proteins using BP as a substrate to generate mutagenic metabolites (cf Table IV), indicating that the substrate specificity of the purified cytochromes is very similar for BP and BP 7,8-dihydrodiol. In all cases, BP 7,8-dihydrodiol was metabolically activated to a more potent mutagen(s) than BP when both substrates were metabolized by the same hemeprotein.

Stereoselective Metabolism of Benzo[a]pyrene 7,8-Dihydrodiol by the Cytochrome P-448 Monoxygenase System. Among the wide array of factors which influence the metabolism of xenobiotic compounds by the microsomal monoxygenase system, enantiomers of optically active compounds have been shown to undergo differential rates of metabolism (45). A study by Huberman et al. (31) on the metabolism of radioactive biosynthetic BP 7,8-dihydrodiol which was diluted with unlabelled racemic diol concluded that greater than 95% of the diol epoxide formed by liver microsomes from 3-methylcholanthrene-pretreated rats corresponded to diol epoxide 2 while Thakker et al. (43) showed that significant amounts of diol epoxides 1 and 2 were formed from racemic BP 7,8-dihydrodiol with liver microsomes as well as by a highly purified monoxygenase system containing either cytochrome P-450 or P-448 (cf Table VIII). These contrasting results prompted studies on the metabolism of the optical isomers of BP 7,8-dihydrodiol. The results presented in Table X demonstrate the marked stereospecificity in the metabolism of optically pure (+)-and (-)-enantiomers of the dihydrodiol by the purified cytochrome P-448-containing monoxygenase system. The highest stereospecificity in the formation of diol epoxide 1 relative to diol epoxide 2 was observed with the (+)-enantiomer of BP 7,8-dihydrodiol. The ratio of diol epoxide 1 to diol epoxide 2 formed was found to be 22:1 with the (+)-enantiomer and 1:4 for the (-)-enantiomer. Both enantiomers of BP 7,8-dihydrodiol were also metabolized to a phenolic derivative (unknown II), tentatively identified as 6,7,8-trihydroxy-7,8-dihydrobenzo[a]pyrene, which accounted for 5-10% of the total metabolites formed. Similar results were also obtained using microsomes from 3-methylcholanthrene-pretreated rats (44). Additional studies have revealed that BP 7,8-dihydrodiol formed from BP by liver microsomes from 3-methylcholanthrene-treated rats is primarily the (-)-enantiomer which rat liver microsomes and the purified monoxygenase system convert mainly into diol epoxide 2 (42,44). Examination of the ability of the purified cytochrome P-448-containing monoxygenase system to convert (+)-and (-)-BP 7,8-dihydrodiol into mutagenic products indicated that both optical isomers of BP 7,8-dihydrodiol are metabolically activated to potent mutagens (43).

Concluding Remarks. One of the primary reasons for determining the mutagenic activity of chemicals is to identify those compounds which may cause cancer. Mutagenic potency may reflect the extent to which compounds are capable of binding to DNA.

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Table X

Stereoselective Metabolism of (-)-and (+)-trans-BP 7,8-dihydrodiol by a Reconstituted Monoxygenase System

Substrate	Metabolites: Tetraols: Trans 1	Cis 1	Trans 2	Cis 2	Unknown[a]: I	II	III	Total
	(nmol formed/nmol hemeprotein/minute)							
(-)-Dihydrodiol	0.14	0.65	3.34	0.19	0.05	0.23	0.05	4.64
(+)-Dihydrodiol	0.46	1.52	0.05	0.00	0.05	0.28	0.18	2.57

Incubation mixtures contained 0.2 nmol of cytochrome P-448, 225 units of NADPH-cytochrome c reductase, 30 µg of phosphatidylcholine, 0.5 µmol of NADPH, 3 µmol $MgCl_2$, 100 µmol of potassium phosphate buffer (pH 6.8) and 40 nmol of radioactive BP 7,8-dihydrodiol in a final volume of 1 ml. Samples were incubated for 10 min at 37°.

[a] Unknowns I and III contain triol-2 and triol-1, respectively, which result from nonenzymatic reduction of diol epoxide 2 and diol epoxide 1 at the 10-position (42,44). Unknown II contains a phenolic metabolite, tentatively identified as 6,7,8-trihydroxy-7,8-dihydrobenzo(a)pyrene (44).

In this system, compounds which are highly susceptible to nucleophilic attack by DNA relative to other types of reactions would be the most potent mutagens. Since the metabolites of BP which are responsible for its mutagenic and carcinogenic activity are anticipated to have high chemical reactivity (low stability) and may be formed in only trace amounts, the formation of such products may not be readily detected by metabolism studies. Because of these considerations, one aspect of our work to identify the ultimate mutagenic and carcinogenic metabolites of BP has centered on the enzymes and mechanisms involved in the metabolic activation of BP and BP derivatives to mutagenic products. We, therefore, developed an enzymatically well-defined model system using the purified monoxygenase system and epoxide hydrase to help elucidate the nature of the ultimate mutagenic metabolite(s) of BP (27). These studies have not only demonstrated the complex interrelationship of both the monoxygenase system and epoxide hydrase in the metabolic activation and inactivation of BP and BP derivatives but have also yielded valuable information concerning the nature of the metabolites responsible for the mutagenicity of BP.

In the absence of epoxide hydrase, the purified cytochrome P-448 containing monoxygenase system metabolizes BP to arene oxides, phenols and quinones (cf Table I). Of twenty-one known and potential primary oxidative metabolites of BP which have been tested for mutagenicity in *S. typhimurium* (21,32,40), only BP 4,5-oxide is a potent mutagen. Thus, BP 4,5-oxide probably accounts for most of the mutations induced when BP is metabolically activated by the purified monoxygenase system in the absence of epoxide hydrase. A small, but significant number of mutations ($\leq$ 20% of the total mutations observed) may be due to the other primary oxidative metabolites of BP which include 6-HOBP.[2] Addition of epoxide hydrase to the purified monoxygenase system decreases mutations in *S. typhimurium* strain TA 98 by a maximum of 30%. The mutations observed when BP is metabolically activated in the presence of epoxide hydrase are not due to BP 4,5-oxide since small amounts of epoxide hydrase completely inactivate BP 4,5-oxide as a bacterial mutagen (cf Figure 2). However, addition of epoxide hydrase to the monoxygenase system results in the formation of BP 7,8-dihydrodiol from BP 7,8-oxide which in turn is metabolically activated by the purified monoxygenase system to the highly mutagenic stereoisomeric BP 7,8-diol-9,10-epoxides which are not substrates for epoxide hydrase. Thus, the effect of epoxide hydrase on the metabolic activation of BP to mutagenic metabolites appears to be a composite of the inactivation of BP 4,5-oxide and the activation of BP 7,8-oxide via BP 7,8-dihydrodiol to BP 7,8-diol-9,10-epoxide.[3] If this is indeed the case, then metabolic activation of 9,10-dihydrobenzo[a]pyrene to mutagenic metabolites should be readily quenched upon addition of epoxide hydrase since this hydrocarbon cannot form a 7,8-diol-9,10-epoxide. Table XI shows

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Table XI

Effect of Epoxide Hydrase on the Metabolic Activation of Benzo(a)pyrene and 9,10-Dihydrobenzo(a)pyrene by a Purified Cytochrome P-448-Containing Monoxygenase System

Substrate	Epoxide Hydrase (units)					
	0	0.5	1.0	2.0	5.0	10.0
	His^{+} Revertants/Plate					
Benzo(a)pyrene	587	517	534	510	510	411
9,10-Dihydrobenzo(a)pyrene	626	506	471	373	251	114

The complete monoxygenase system consisted of 50 μg of phosphatidylcholine, 150 units of NADPH-cytochrome c reductase, 0.1 nmol of cytochrome P-448, 0.1 μmol of NADPH, 12.5 nmol of substrate and 0-10 units of purified epoxide hydrase in a final volume of 0.5 ml containing 2 x 10^8 bacteria of strain TA98. One unit of epoxide hydrase is defined as 1 nmol styrene glycol formed per min from styrene oxide.

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that 9,10-dihydrobenzo(a)pyrene is metabolically activated to mutagenic metabolites by the purified monoxygenase system and that addition of epoxide hydrase results in a greater than 85% inhibition in the mutations observed.

Future studies with the purified monoxygenase system and epoxide hydrase are aimed at predicting the ultimate mutagenic metabolite(s) of other polycyclic aromatic hydrocarbons. Indeed, preliminary results in our laboratory (47) have shown that benzo(a)anthracene 3,4-dihydrodiol is metabolically activated to a more potent mutagen(s) by the purified cytochrome P-448 containing monoxygenase system than is benzo(a)anthracene, or benzo(a)anthracene 1,2-, 5,6-, 8,9- or 10,11-dihydrodiol. A 1,2-epoxide of the 3,4-dihydrodiol would be in the "bay region" of the hydrocarbon. Such "bay region" epoxides have been predicted to be the most biologically active metabolites of polycyclic aromatic hydrocarbons based on correlations of known carcinogenicity (48) and on quantum mechanical calculations (49). We are now evaluating the possibility that the stereoisomeric benzo(a)anthracene 3,4-diol-1,2-epoxides may be the ultimate carcinogenic metabolites formed from benzo(a)anthracene.

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Footnotes

1 Abbreviations used: BP, benzo(a)pyrene; 1-HOBP, 1-hydroxybenzo(a)pyrene; 2- to 12-HOBP, other isomeric phenols; BP 7,8-dihydrodiol, (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene; BP 4,5-, 9,10- and 11,12-dihydrodiol, other dihydrodiols of BP; BP 7,8-diol-9,10-epoxide, either or both stereoisomers of (±)-7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydro BP; diol epoxide 1, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydro BP; diol epoxide 2, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro BP; H_4-9,10-epoxide, (±)-9,10-epoxy-7,8,9,10-tetrahydro BP.

2 6-HOBP oxidizes nonenzymatically to 1,6-, 3,6- and 6,12-quinone (46). If all of quinone fraction 1 (cf Table 1) were due to formation of 6-HOBP, then approximately 10-15% of the total mutations observed when BP is metabolized by the cytochrome P-448-containing monoxygenase system could be due to 6-HOBP.

3 Based on the observation that BP is metabolized predominantly to the (-)enantiomer of trans-BP 7,8-dihydrodiol by liver microsomes from 3-methylcholanthrene-treated rats (42,44) and the (-)enantiomer is metabolized predominantly to diol epoxide 2 (cf Table VIII), most of the mutagenic activity of metabolically activated BP 7,8-dihydrodiol must be due to diol epoxide 2 using the reconstituted monoxygenase system.

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7

In vitro Reactions of the Diastereomeric 9,10-Epoxides of (+) and (−)-*trans*-7,8-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene with Polyguanylic Acid and Evidence for Formation of an Enantiomer of Each Diastereomeric 9,10-Epoxide from Benzo[*a*]pyrene in Mouse Skin

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Benzo[*a*]pyrene (BP) is a potent carcinogen which is widespread in the environment. The precise mechanism by which BP and other polycyclic aromatic hydrocarbons (PAH's) induce neoplasia is not presently known. There is, however, some correlation between the extent of binding of PAH's to cellular macromolecules (DNA, RNA, and protein) and their carcinogenicity (1-3). Since carcinogenic PAH's are also mutagenic in mammalian and bacterial test systems in which they undergo metabolic activation (3-5), their interactions with nucleic acids are of obvious biological importance. In addition, these interactions with nucleic acids are prospective initiation events in chemical carcinogenesis. BP is irreversibly bound to DNA *in vitro* when a cytochrome P-450 monooxygenase system is present (6,7). A number of reactive metabolites have been considered. These include various epoxides and arene oxides (3,8,9), free radicals (10,11), radical cations (12, 13), and "non-K-region" diol epoxides. Recent studies on the binding of *trans*-7,8-dihydroxy-7,8-dihydro-BP (BP 7,8-dihydrodiol) to DNA *in vitro* (14) and evidence that this binding is mediated by a 7,8-dihydrodiol-9,10-epoxide (9) have led to the synthesis of the two possible diastereomers (15,16) and to extensive study of their interactions with nucleic acids (9,14,17-26).

The two possible racemic diastereomers in which the benzylic hydroxyl group and the oxirane oxygen are either cis, 1 or trans, 2 are highly reactive (Figure 1). In water, these diol epoxides are rapidly hydrolyzed by cis and trans addition of water to the epoxides at C-10 (27-30). That 1 and/or 2 are probable ultimate carcinogenic forms of BP is suggested by the high carcinogenicity of their metabolic precursors, BP 7,8-oxide and BP 7,8-dihydro-

diol (31,32) as well as by their high mutagenicity toward mammalian and bacterial cells (30,33-36). The high chemical reactivity and the mutagenic activity provide clear evidence of the ability of these diol epoxides to damage nucleic acids.

Modification of nucleic acids with carcinogens and mutagens has been extensively studied (37). For example, almost every position on the nucleosides as well as the phosphate backbone is known to be alkylated to some extent. Common reactions are at N^7 of guanine 6a (Figure 2) and N^3 of adenine (38) (see Table I). Recent quantitative studies have revealed that the amount of O-alkylation at O^6 of guanine (39,40) and at phosphate groups to form phosphotriesters (41,42) correlates better with the biological activity of the alkylating agents than does the total extent of alkylation. Presently it is unclear whether a specific alkylation is important to carcinogenicity or whether other factors such as differences in the rate of DNA repair (N^7 > O^6-alkyl) are responsible for this effect (43).

Extensive methylation of DNA at N^7 of guanine by *N*-methyl-*N*-nitrosourea is typical of methylating agents. Reactive aromatic compounds of higher molecular weight show different selectivity. For example, *N*-acetoxy-2-acetylaminofluorene 3 reacts selectively at C^8 of guanosine 6b (44) and to a lesser degree at the 2-amino group of guanosine (45). 7-Bromomethylbenz[*a*]anthracene 4 (46) and 7,12-dimethylbenz[*a*]anthracene 5,6-oxide 5 (47) alkylate predominantly at the exocyclic 2-amino group of guanosine. Prior orientation of the planar reactant by intercalation between the base pairs may account for selective alkylation of the 2-amino group (48). Intercalation into nucleic acids also tends to dramatically increase the solubility of PAH's in aqueous solutions (49,50). Thus, intercalation of PAH derivatives may serve the dual function of increasing the effective concentration of the reactive form of the PAH near the nucleic acid and of orientating this reactive form for specific sites of the polymer.

Diol epoxides 1 and 2 have been shown to attack the exocyclic 2-amino group of guanosine in nucleic acids. Diol epoxide 2, on reaction with polyguanylic acid (poly (G)) in 75% acetone/water, has been shown to form a pair of diastereomeric adducts by trans opening of the epoxide at C-10 (22). One of these diastereomers and other unidentified products were detected in RNA isolated from bovine bronchial explants which had been exposed to [^{3}H]-BP (23). Diol epoxide 1 has been shown to form both cis and trans adducts at C-10 with the 2-amino group of guanosine in poly (G) (50% acetone/water) as well as alkylate the phosphate backbone to form phosphotriesters (26). The products formed from the *in vitro* reactions of the diol epoxides with DNA have been studied (9,18,23,25).

We describe here details of the reactions of diol epoxides 1 and 2 with poly (G) and provide evidence for the structure of the RNA adducts which form when [^{3}H]-BP is painted on mouse skin. Adducts from both diol epoxides 1 and 2 are formed in this *in vivo*

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Figure 1. Hydrolysis of diol epoxides to tetraols.

Unless specifically designated, diol epoxide 1 and diol epoxide 2 refer to racemic compounds. The structures 1 and 2 are intended to show the relative stereochemistry between the two sets of diastereomers and to show the absolute stereochemistry of the diol epoxide products derived from the (−) enantiomer of BP 7,8-dihydrodiol.

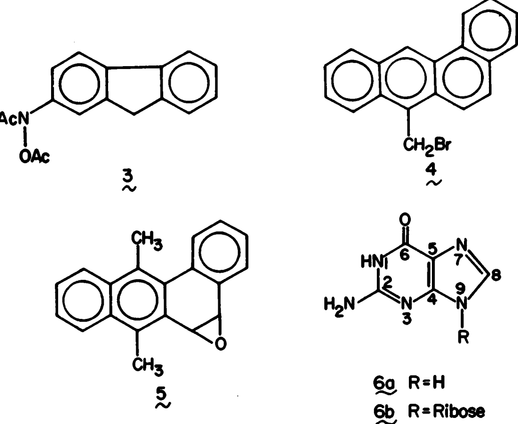

Figure 2. Reactive aromatic compounds and guanosine

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experiment with a tissue toward which BP is carcinogenic. These adducts are optically active and their absolute stereochemistry has been assigned through the use of the optically pure enantiomers of diol epoxides 1 and 2.

Materials and Methods

Diol Epoxides. Diol epoxide 1 was synthesized from BP 7,8-dihydrodiol via a bromo-triol, while diol epoxide 2 was obtained by *m*-chloroperbenzoic acid oxidation of the same BP 7,8-dihydrodiol (15,16). Tritiated diol epoxides were synthesized from [9, 10-^{3}H]-BP 7,8-dihydrodiol as previously described (27) and were used at a specific activity of 2.3 μCi/μmol. Optically active diol epoxides 1 and 2 were synthesized from (+) and (-)-BP 7,8-dihydrodiol which had been resolved via the diastereomeric bis-(-)-α-methoxy-α-trifluoromethylphenylacetates (51-53).

Binding of Diol Epoxides to Poly (G). The potassium salt of polyguanylic acid (5') (mol. wt. > 150,000) was obtained from Sigma. [8-^{3}H]-Polyguanylic acid was purchased from Miles Research Products and used at a specific activity of 0.31 μCi/μmol of phosphorous. For a typical binding experiment, 1.7 mg of poly (G) was dissolved in 0.5 ml of distilled water and the pH was adjusted by addition of dilute HCl or KOH. A pH of 7.0 (monitored with a pH electrode) was used in all cases except those specifically designated. An equal volume of spectroscopic grade acetone was added and the solution was treated with 0.4 mg of either diol epoxide in 100 μl of dry tetrahydrofuran (THF). The solutions were incubated at 37°C for 18 hours. Tetraols which result from hydrolysis were removed by extraction with three equal volumes of ethyl acetate. The modified polymer was isolated by acidification of the solution with 0.1 volume of 2.5 M sodium acetate (pH 5.0) and precipitation of the polymer with 3 volumes of ethanol. The mixture was stored at 0°C overnight and then centrifuged for 15 min. at 3,000 rpm. The supernatant was removed and the precipitated polymer was washed with 2.0 ml of ethanol. The polymer could be redissolved in water and the precipitation procedure was repeated without significant change in the amount of bound hydrocarbon absorbing at 350 nm (54).

Hydrolysis of Poly (G) to Nucleosides. Modified poly (G) was dissolved in 1.0 N KOH and incubated for 18 hours at 37°C. The solution was neutralized with 2 N $HClO_4$ which resulted in precipitation of $KClO_4$. After centrifugation the salt was separated by decanting the aqueous solution. Tris-(hydroxymethyl)-aminomethane was added to the aqueous supernatant to produce a final concentration of 1.0 mg/ml and the pH was adjusted to 8.4. Bacterial alkaline phosphatase (1 unit/mg of poly (G)) was added and the solution was incubated for 24 hours at 37°C, which affected complete hydrolysis to nucleosides as monitored by paper chromatography.

Removal of Ribose by N^7-Methylation with Dimethyl Sulfate. After base hydrolysis, the modified nucleotide mixture from 50 mg of poly (G) was dissolved in 10 ml of 2 M KH_2PO_4 at pH 7.0 and

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treated with three 100 μl aliquots of dimethyl sulfate at 2 hour intervals. The pH was maintained > 6.0 by occasional addition of dilute KOH. When the uv spectrum of the mixture showed the spectral shifts with pH which are characteristic of 7-methylguanosine (55), the pH was adjusted to 6.0 and the mixture was refluxed for 1 hour to remove the ribose from the methylated base. The diol epoxide-7-methylguanine base was selectively extracted with three equal volumes of ethyl acetate.

Treatment of Mice with [^{3}H]-BP. The backs of eleven C57BL/6J female mice were shaved and, two days later each mouse was painted with 100 μg of [^{3}H]-BP (Amersham Searle) (1.4 mCi/mouse) in 100 μl of acetone. Twenty four hours later the mice were sacrificed, and the epidermal layer was isolated and homogenized as described (56). The RNA was isolated by a phenol-cresol method (57). The 1.3 mg of RNA thus obtained contained ≈ 2.8 x 10^5 dpm/mg.

High-pressure Liquid Chromatography. The nucleoside adducts from 1 and 2 were conveniently separated from unmodified guanosine with a Poragel PN column (3/8" x 3') eluted with 85% methanol in water at a flow rate of 5.0 ml/min. For high resolution analysis of the nucleoside adducts, a Waters μC_{18}-Bondapak column (1/4" x 1') was eluted at a constant rate of 1.2 ml/min with 39% methanol/water for 50 minutes followed by a 60 minute linear gradient to 50% methanol/water.

Results

At pH 7.0 diol epoxides 1 and 2 were found to bind to poly (G) to about the same extent. Binding was characterized by the uv absorption pattern of the 7,8,9,10-tetrahydro-BP moiety in the 320 - 355 nm region (Figure 3). The absorption maximum at 352 nm represents a bathochromic shift of 9 nm and a substantial decrease in the extinction coefficient which would be expected for the 7,8,9,10-tetrahydro-BP chromophore in this region. These changes in the chromophore largely disappear upon alkaline hydrolysis of the modified poly (G) (inset Figure 3). This clearly indicates that the hydrocarbon is closely associated with the polymer (50). Evidence proving that this association involves covalent bond formation is presented later.

Effect of pH on Rate and Extent of Binding. The rate and extent of binding were explored with [9,10-^{3}H]-diol epoxides since total binding could be accurately determined radiochemically. Buffer solutions were avoided since the diol epoxides react with phosphate ions. For example, from 5 - 10% of the hydrocarbon becomes non-extractable due to alkylation of 0.05 M phosphate at pH 7.0 (26). When these phosphate-diol epoxide products were heated for 1 hour on a steam bath, all of the hydrocarbon was liberated from its phosphate esters. When diol epoxide was added to pure water only a trace (<< 1%) of hydrocarbon remained after extraction, which indicates that very little non-extractable solvolysis products are formed.

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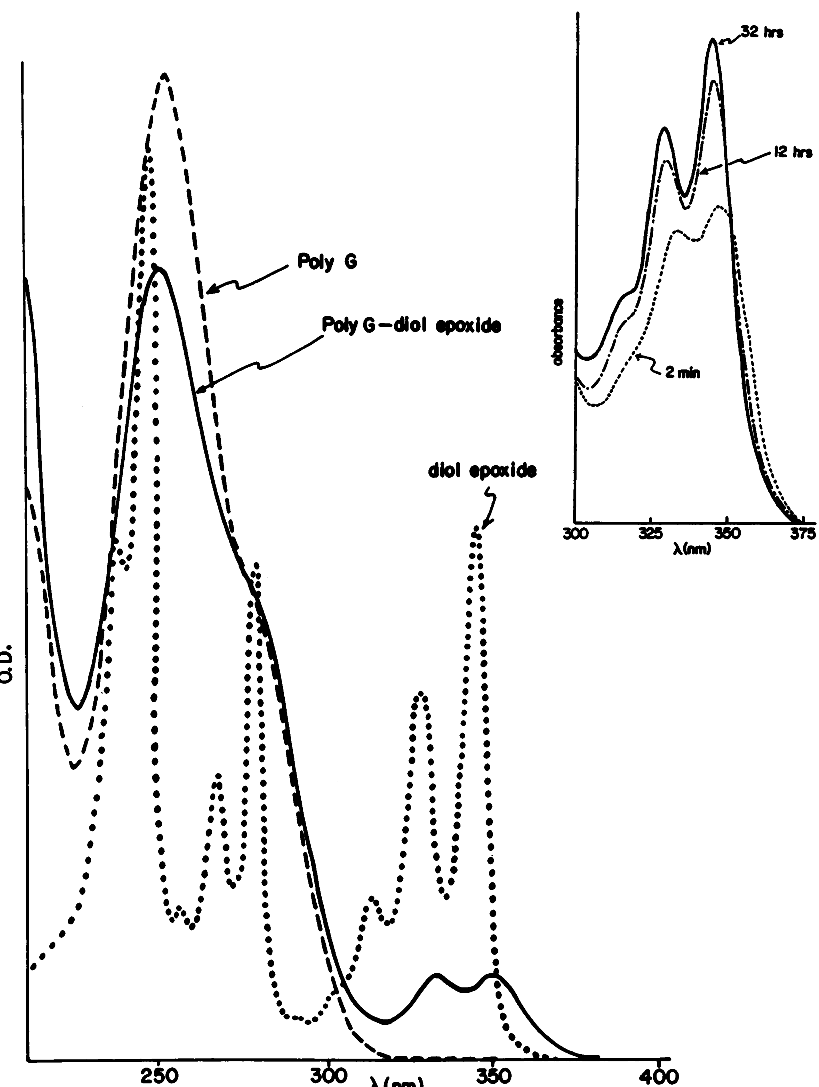

Figure 3. Ultraviolet spectra of poly (G), diol epoxide 1, and poly (G) modified by diol epoxide 1. The following ε values were observed, poly (G) in water ϵ_{260} = 9,000 and diol epoxide 1 in dry THF ϵ_{343} = 46,500. The inset shows repetitive scans of poly (G)-diol epoxide 1 in 0.5N KOH at 25°C.

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The effect of pH on total binding is shown in Figure 4. Maximum binding takes place in slightly acidic media. Both diol epoxides showed similar pH-dependent rates of binding to poly (G). Binding of diol epoxide 1 was slightly faster but qualitatively the same as that of diol epoxide 2. Figure 5 shows the rate of binding for diol epoxide 2 at various pH values. The rate of binding at pH 4.0 is at least twenty times greater than that at pH 7.0. Even though binding at pH 4.0 is much faster than that at pH 7.0, the total amount bound after 2 hours of incubation is slightly less. These observations are consistent with acid catalyzed alkylation of the polymer competing with acid catalyzed hydrolysis of the diol epoxide to tetraols.

Reaction on the Phosphate Backbone of Poly (G)

When poly (G) was modified by either diol epoxide and purified by solvent extraction and multiple precipitation, non-bound tetraols were completely removed. The uv spectrum of this purified modified poly (G) showed a distinct valley at 343 nm. When the modified polymer was heated to 100°C at neutral pH a small amount of free tetraols was liberated and could be detected by their uv spectrum. The liberation of tetraols was confirmed by co-chromatography with standards and by the mass spectrum of the tetraacetyl derivative (M^+ at m/e 488). After extraction of these tetraols prolonged heating (4 hours) of the modified poly (G) failed to liberate further quantities of tetraols. Base hydrolysis of modified poly (G) which had not been heated in water also led to the formation of tetraols. Quantitative measurements indicated that 10 - 15% of the total bound hydrocarbon could be released as tetraols.

Since polycyclic aromatic hydrocarbons, such as the tetraols or the diol epoxides themselves, can physically intercalate into nucleic acids the possible presence of residual non-covalently-linked hydrocarbon derivatives cannot, *a priori*, be eliminated. Addition of poly (G) modified by 1 to ^{18}O enriched water (18%) followed by heating for 15 minutes at 100°C generated tetraols which incorporated 0.96 atom % of solvent oxygen. When *cis*-1 and *trans*-1 tetraols were added to this same poly (G) solution and similarly treated, no incorporation of ^{18}O in the reisolated tetraols was observed. This result strongly argues against the presence of physically bound tetraols. Since the half-life for binding is less than 10 minutes at pH 7.0, it seems unlikely that intact diol epoxides could have survived during the incubation without either binding to the poly (G) or undergoing hydrolysis to extractable tetraols. In addition, it is unlikely that the diol epoxides could selectively resist extraction compared to tetraols. Relative amounts of cis and trans tetraols which form in various conditions are shown in Table II. Base hydrolysis of diol epoxide 1 leads to substantial *trans*-1 tetraol while the tetraols gener-

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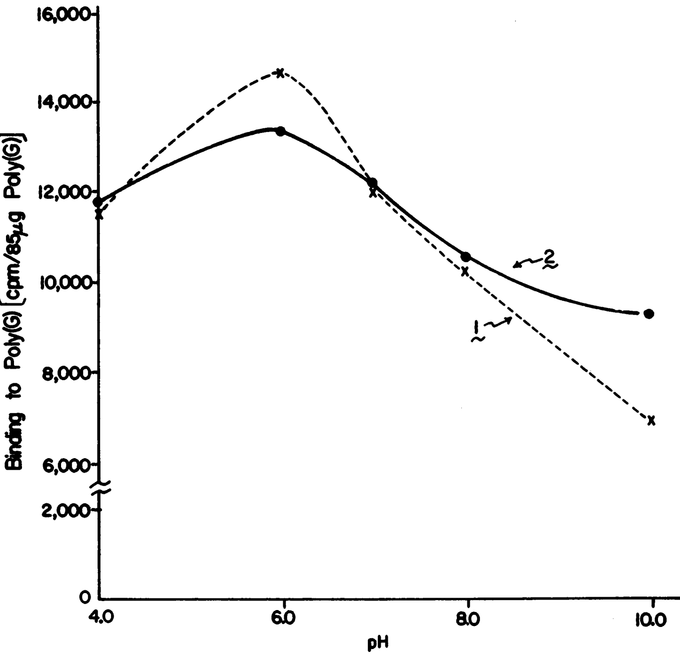

Figure 4. Extent of binding as a function of pH.

Poly (G) solutions of 3.4 mg/ml were adjusted to pH 4.0, 6.0, 7.0, 8.0, and 10.0, then diluted with an equal volume of acetone. 2.95 μCi [9,10-^3H] diol epoxide 1 or 2 was added to 1.0 ml of the stock poly (G) solutions at 37°C, and 50-μl aliquots of the reaction mixture were removed at convenient time intervals. Each aliquot was added to a tube containing 1.0 ml of H_2O and 5.0 ml of ethyl acetate. This mixture was shaken vigorously, and the ethyl acetate was removed. The aqueous solution of poly (G) was then extracted twice with 5.0 ml of ethyl acetate. Since tetraols are readily extracted from aqueous poly(G) solutions the [^3H] counts present were assumed to be bound to poly (G). Results obtained when uv absorbance at 350 nm was used to quantitate binding indicated that the above extraction procedure was correct to ±10%. Diol epoxide 1 was examined after 60 min of reaction and diol epoxide 2 after 50 min of reaction. Points represent the average of two separate determinations.

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Table I. Methylation of Salmon Sperm DNA by *N*-Methyl-*N*-nitrosourea. Data of Lawley and Shah (38).

Base	Site	% Total identified methylation of bases
Guanine	N^7	75.7
	O^6	7.3
	N^3	1.1
Adenine	N^3	11.2
	N^1	1.4
	N^7	2.5
Thymidine	N^3	0.3
	O^4	0.1
Cytidine	N^3	0.6

Table II. Percent cis and trans Tetraols from Diol Epoxides 1 and 2 and Modified Poly (G).

Source	Conditions	*cis*-1	*trans*-1	*cis*-2	*trans*-2
1	50% acetone/water, pH 7.0 (conditions of binding)	85	15		
2	50% acetone/water, pH 7.0 (conditions of binding)			15	85
Poly (G) - 1	100°C, pH 7.0, 15 minutes	75	25		
Poly (G) - 2	100°C, pH 7.0, 15 minutes			39	61
1	1.0 N KOH, 20% THF/H_2O, 37°C, 24 hrs.	18	81		
Poly (G) - 1	1.0 N KOH, 37°C, 24 hrs.	95	5		
Poly (G) - 2	1.0 N KOH, 37°C, 24 hrs.			2	98

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ated from base hydrolysis of poly (G) modified by 1 are almost exclusively *cis*-1 tetraols. This provides another argument that the labile hydrocarbon products are not due to residual diol epoxides.

These results are consistent with the presence of a heat and alkaline labile covalent diol epoxide adduct. The formation of phosphate-diol epoxide monoesters which could be cleaved by simple heating at pH 7.0 together with the chemical and thermal stability of the guanosine adducts discussed below strongly suggest that the liberated tetraols are derived from alkyl phosphates of poly (G) (37,40-42,58).

Reaction on the Guanine Base of Poly (G). When modified poly (G) was hydrolyzed in base, the tetraols released from the phosphate adducts were readily removed by extraction of the neutralized hydrolysate with ethyl acetate. After alkaline phosphatase treatment, the nucleoside adducts became sufficiently nonpolar to allow extraction into organic solvents. However, the nucleoside adducts were more conveniently isolated by reverse phase chromatography of the hydrolysate on a Poragel PN high-pressure liquid chromatography column (Figure 6). Guanosine, nucleoside adducts, and tetraols are all separated. The high capacity of the column allows separation of 10 - 100 mg samples with a single injection. The nucleoside adduct fraction contains > 95% of all non-tetraol-hydrocarbon products.

High resolution chromatography on μC_{18}-Bondapak separated the nucleoside adduct fraction into a number of peaks. Each diol epoxide formed four products which, on the basis of peak area, appeared to consist of two groups. Nucleoside-diol epoxide 1 products were eluted in the approximate ratio of 1:2:1:2. Products from diol epoxide 2 were overall slightly more polar and eluted in ratio of 3:1:1:3 (Figure 7). The CD spectra (Figure 8) of the individual peaks established that the products of equal peak area were actually pairs of diastereomers which resulted from the reaction of racemic diol epoxides with optically pure poly (G). Further proof that the pairs of products are diastereomers was obtained through the use of optically pure diol epoxides 1 and 2, since only one component of each pair was obtained from each of the diol epoxide enantiomers (Table III). Thus, when (+)-diol epoxide 1 derived from (+)-BP 7,8-dihydrodiol was reacted with poly (G), only nucleoside adducts F and D were formed. Similarly, (+)-diol epoxide 2 from (-)-BP 7,8-dihydrodiol produced only nucleoside adducts B and G.

Structure of the Guanosine Adducts. Normally the position at which a purine base is alkylated can readily be established by uv spectroscopy. Characteristic changes in the uv spectra of the alkylated bases as a function of pH generally provides sufficient evidence for assignment of the site of alkylation (37). This approach proved to be of limited value in the present study since the uv spectra of the nucleoside adducts as a function of pH showed little change. The absorption spectrum of the 7,8,9,10-

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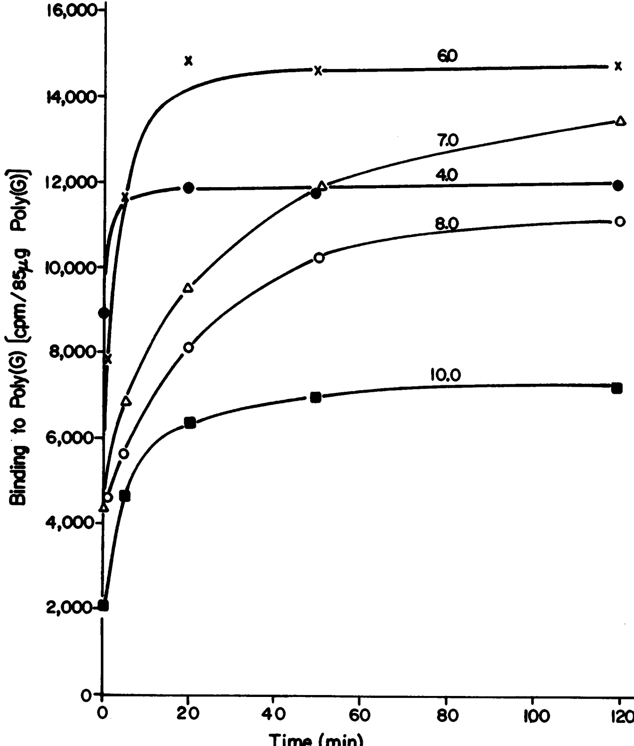

Figure 5. Rate of binding of diol epoxide 2 to poly (G) as a function of pH. Conditions were the same as in Figure 4 with the exception that the binding was examined at the indicated time intervals.

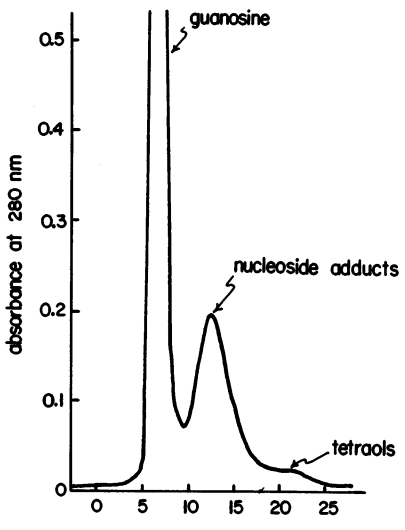

Figure 6. Purification of guanosine-diol epoxide 1 products via Poragel PN chromatography (see "Materials and Methods" for details)

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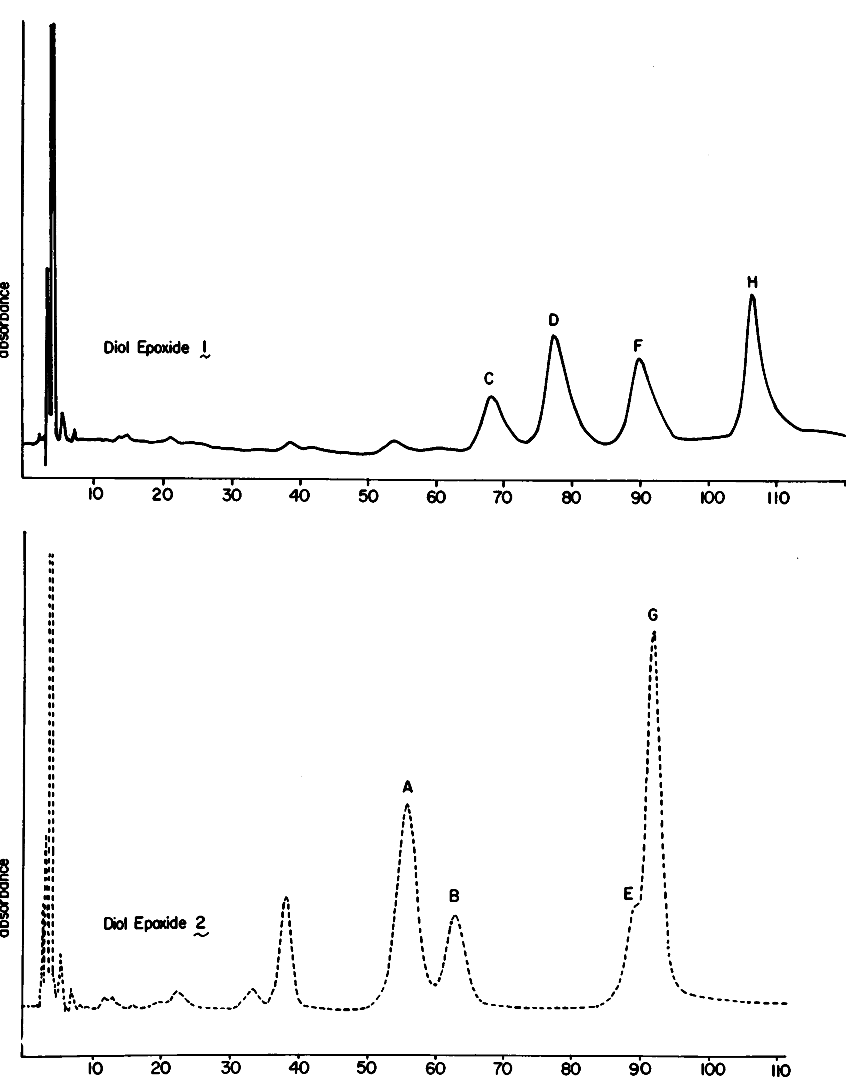

Figure 7. High resolution chromatography of the guanosine adducts from diol epoxides 1 and 2. Conditions are as described in "Materials and Methods." The peak with a retention time of 39 min in the lower trace is caused by trans-2 tetraol from diol epoxide 2 which was a contaminate in this sample.

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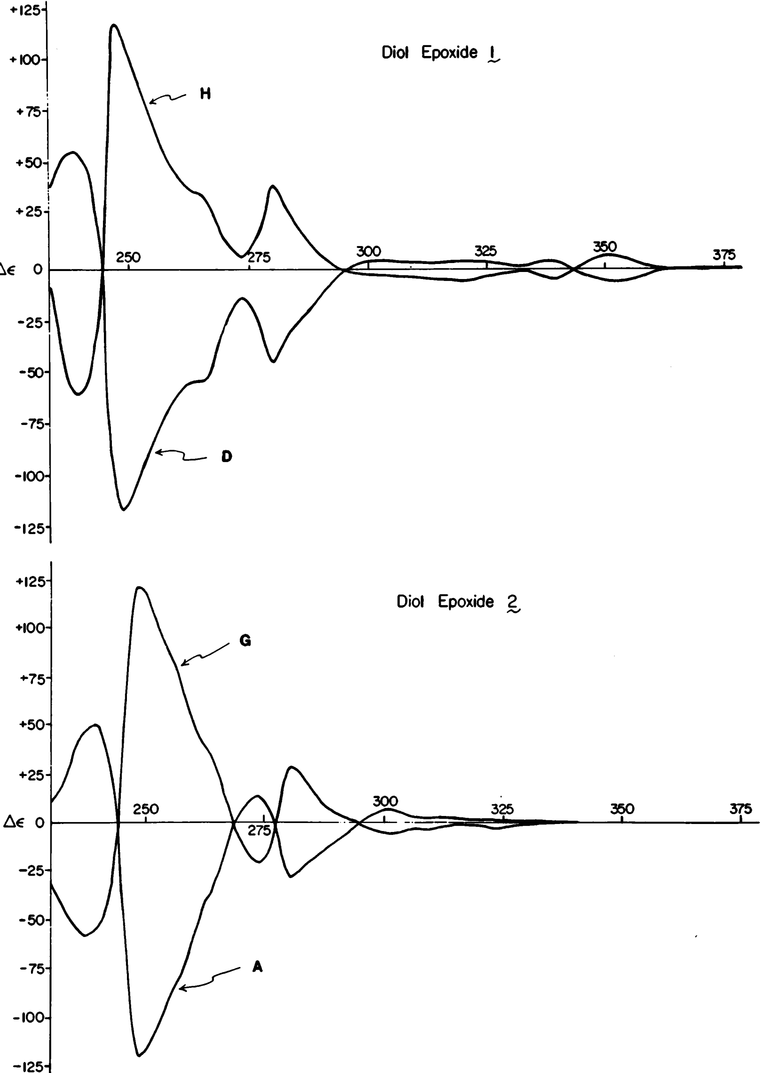

Figure 8. CD spectra of the major pairs of guanosine adducts from diol epoxide 1 (D and H) and diol epoxide 2 (A and G).

Spectra were determined in methanol, and $\Delta\varepsilon$ is based on $\varepsilon_{344} = 37,000$. Both sets of products were isolated as in Figure 7 and will be shown later to arise from trans addition of the 2-amino group of guanosine at C-10 of the respective diol epoxide. The two pairs of diastereomeric cis addition products show almost identical mirror image CD spectra similar to D and H.

Table III. Relation between the Nucleoside Adducts and the Optically Active BP 7,8-Dihydrodiol and Diol Epoxide Products from Which They Are Derived.

BP 7,8-Dihydrodiol[a]	Diol Epoxide[a]	Nucleoside Adduct[b]	Ret. Time (min)[e]	Symbol[e]
(+)-dihydrodiol	(+)-diol epoxide 1	(+)-*cis*[c,d]	92	F
		(-)-*trans*[c]	79	D
(-)-dihydrodiol	(-)-diol epoxide 1	(-)-*cis*	70	C
		(+)-*trans*	108	H
(+)-dihydrodiol	(-)-diol epoxide 2	(+)-*cis*	89	E
		(-)-*trans*	56	A
(-)-dihydrodiol	(+)-diol epoxide 2	(-)-*cis*[c]	64	B
		(+)-*trans*[cd]	93	G

[a] Sign of $[\alpha]_D$.
[b] Sign of CD Cotton effect at 250 nm.
[c] Nucleoside adducts identified in the RNA isolated from the skin of mice which had been treated with BP.
[d] Due to co-chromatography, nucleoside products F and G can not be distinguished.
[e] Chromatographic profile of nucleoside adducts (see Figure 7).

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tetrahydro-BP chromophore, whose extinction coefficient at 270 nm is almost 10 times as large as that of guanosine (Figure 9), masked changes that presumably occur in the purine chromophore on changing pH. In addition, the absorption spectrum near 340 nm, which is due entirely to the hydrocarbon portion of the adduct, was observed to change with pH. Difference uv spectra between the nucleoside adducts and the tetraols provided little help since the absorption pattern in the nucleoside adducts is slightly shifted compared to that of tetraols. Without the conventional spectral method for assignment of the position of alkylation, chemical methods were used to locate the site of substitution on the guanine base.

Acid Hydrolysis of the Nucleoside Adducts. When the diastereomeric mixture of purified nucleoside adducts from 1 was heated in 0.1 N HCl, two tetraols (*cis*-1 and *trans*-1) and guanosine were rapidly generated. At 85°C the half-life for release of tetraols is approximately 15 minutes. When these nucleoside adducts were treated with 0.1 N HCl in ^{18}O enriched water (18%) at 85°C, mass spectral analysis of the tetraol mixture after acetylation showed incorporation of 0.96 atom % ^{18}O into the isolated tetraols. Overall recovery of the tetraols exceeded 80%. Under these conditions the tetraols, *cis*-1 and *trans*-1, incorporated 0.86 atom % of one solvent oxygen (26). Since a carbon-carbon bond would not be expected to be acid-labile, the experiment suggest an oxygen or nitrogen to carbon bond in the site of attachment.

Reaction of Diol Epoxides 1 and 2 with [8-^3H]-Poly (G). At pH 7.0 the most reactive site on the guanosine base towards alkylating reagents is generally the N^7 position (37). After methylation at N^7 the hydrogen at C^8 becomes readily exchangeable with water (59). Thus, treatment of [8-^3H]-poly (G) with diol epoxides 1 or 2 would provide a sensitive test for C^8 or N^7 alkylation.

Binding of the diol epoxides 1 and 2 with [8-^3H]-poly (G) was performed in 50% acetone/water at pH 7.0. After incubation overnight, the solutions were diluted with 5 volumes of pH 7.0 phosphate buffer, and the water was recovered by distillation at 25°C under high vacuum. Release of tritium above the blank of 0.5% was not observed for either diol epoxide despite modification of 10% of the guanosine sites. The residual modified poly (G) was hydrolyzed to nucleosides which were purified by Poragel PN chromatography. These nucleoside adducts had a specific activity which was > 80% of that of the starting guanosine in [8-^3H]-poly (G).

This result is consistent with alkylation at a site other than at C^8 or N^7 of guanosine. However, exchange of the C^8 hydrogen of 7-methylguanosine relies on the stability of the 7-methylimmonium ion. The stability of the highly hindered immonium ion which would form if the diol epoxides reacted at N^7 is presently unknown. The hypothetical pathway in Figure 10 follows the proposed route of alkaline (pH 9 - 10) decomposition of 7-methylguanosine and would result in retention of tritium at C^8. Strong

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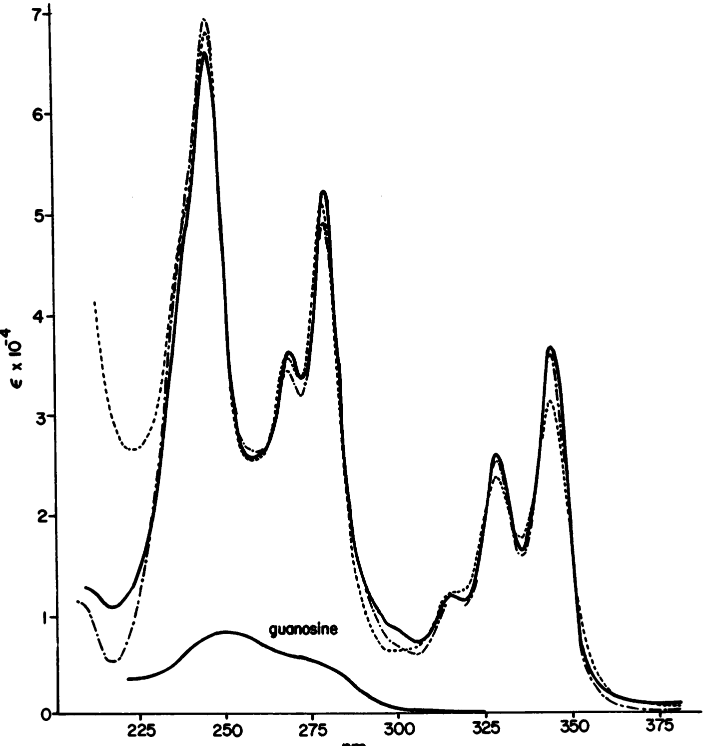

Figure 9. Ultraviolet spectra of nucleoside adducts from diol epoxide 1 in methanol as a function of pH. Neutral (——); alkaline (— · —); acidic (– – –). The ε values of the adduct mixture are based on the specific activity of the [9,10-^{3}H] diol epoxides.

DEBP N7 N9 ^{3}H 8 Ribose — fast? pH 7.0 → DEBP N OH ^{3}H N R ⇌ DEBP N–H O N–C–^{3}H 9 R + DEBP N–C–^{3}H 7 O N–H R

no exchange of ^{3}H

1.0N KOH 100°C

DEBP N–H NH$_2$ + K$^+$ $^-$O–C(=O)–^{3}H

Figure 10. A possible decomposition pathway of N^7-substituted guanosine leading to retention of tritium in the product. DEBP represents diol epoxide 1 or 2 after opening of the 9,10-epoxide at C-10.

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base treatment of the mixture of formamides would produce the non-volatile salt of [1-^{3}H]-formic acid (60). To test for this possibility, the purified nucleoside adducts from [8-^{3}H]-poly (G) were heated at 100°C in 2 N KOH, conditions where [8-^{3}H]-guanosine exchanges. Most of the tritium distilled from this basic solution. After acidification and redistillation volatile [1-^{3}H]-formic acid was not found in the distillate. The failure to release volatile [1-^{3}H]-formic acid upon acidification eliminated alkylation at N^7 and the mechanism in Figure 10. Taken together, these results completely exclude N^7 and C^8 as the site of attachment of the hydrocarbon.

Base Stability of Nucleoside Adducts. In 1.0 N KOH at 100°C certain (Table IV) substituted purine ribosides rapidly degrade. This degradation can proceed by attack of hydroxide on the imidazole ring at C^8 or at various sites of the pyrimidine ring. However, purine ribosides with a hydrogen on the N^1 position, except N^7 derivatives, are deprotonated to yield a monoanion and become stabilized towards further reaction with base (60). Accordingly, N^1, N^3, N^7, and O^6 substituted guanosines are known to be degraded (Table IV) while N^2 substituted guanosine is stable towards base treatment (61,62). Treatment of nucleoside adducts with 1 N KOH at 100°C for 2 hours resulted in only minor degradation and allowed high recovery of unchanged adducts. This strongly suggests the presence of an amidic proton in the nucleoside adducts.

Determination of the pK_a Values of the Nucleoside Adducts. Measurement of the pK_a's of the nucleoside adducts could establish whether or not a free amidic proton is present at N^1. Spectrophotomeric titration was possible but subject to uncertainty due to the small change in the uv spectrum that occurred upon ionization. Since the sample size precluded direct titration, a more sensitive and reliable method was sought. The nucleoside adducts are soluble in water but can be extracted into polar organic solvants. Since the ionized form of guanosine is much more soluble in water than the neutral form, a pK_a should be detectable by a change in partition coefficient with pH (26,63) (Figure 11). The acidic pK_a at 9.8 indicates deprotonation from neutral nitrogen and the basic pK_a at 1.5 indicates protonation of neutral nitrogen. Although the pK_a values obtained are approximate due to the selective removal of one component from the acid-base equilibrium, the presence of a free N^1 proton is clearly demonstrated. This result, in combination with the stable nature of the nucleoside adducts towards base treatment, eliminates N^1, N^3, and O^6 as possible sites of attachment. Since N^7 and C^8 substitution had previously been eliminated, an N^2 substituted guanosine becomes the sole possible structure for the nucleoside adducts.

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Table IV. Effect of 1.0 N KOH at 100°C on Substituted Guanosines.

Compound	Effect
	degraded
	stable
	degraded at C^2
	degraded at O^6
	degraded at C^8
	stable

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Nmr and Mass Spectra of the Adducts. Final determination of the structures of the nucleoside adducts from 1 by mass spectrometry and proton nmr was greatly handicapped by the added mass and the interfering resonances of the ribose moiety. Since acid treatment cleaved the hydrocarbon-guanosine bond much more readily than the glycosidic linkage, the normal method of direct depurination with acid was not useful. An alternative mild method for cleavage of the glycosidic linkage was sought. Since the N^7 position was known not to be substituted and since dimethyl sulfate shows a marked preference for alkylation of N^7, the mixture of four nucleotide adducts obtained from diol epoxide 1 was treated with dimethyl sulfate to labilize the ribose linkage (55) (Figure 12).

Mass spectrometry of the derived triacetate of the methylated adducts by chemical ionization with methane showed that 7-methylguanine-tetrahydro-BP triacetates had been formed. Chromatography on ODS allowed the isolation of two products (Figure 13) 8 and 9 with identical mass spectra (26). Only two adducts were expected since loss of the optically pure ribose moiety reduces each pair of optically active diastereomers with mirror image CD spectra into single racemic compounds. Fourier transform 220 MHz proton nmr of the major product showed three acetyl singlets at 1.98, 2.04, and 2.25, one broad N-methyl singlet at 3.38, the four one proton signals from positions 7,8,9, and 10 of the tetrahydro-BP ring at 6.71, 5.52, 5.58, and 6.14, respectively, and the remaining C^8 and pyrene signals from 7.95 to 8.28 ppm. Comparison of the chemical shifts and coupling constants of this product ($^3J_{7_{eq},8_{eq}}$ = 5.2, $^3J_{8_{eq},9_{eq}}$ = 5.4, $^3J_{9_{eq},10_{eq}}$ = 2.6 Hz) with those of the 9,10-*trans*-aniline adduct (--NHC_6H_5 at C-10 of 8) from diol epoxide 1 (28) convincingly established that the major product was derived from trans addition of the 2-amino group of guanine to the C-10 position of 1. Similarly, the proton nmr of the minor product showed three acetyl singlets at 1.95, 2.00, and 2.13, one N-methyl singlet at 3.93, the four one proton signals from positions 7,8,9, and 10 of the tetrahydro-BP ring at 6.93, 6.18, 5.61, and 6.29, respectively, and the remaining C^8 and pyrene signals from 7.95 to 8.40 ppm. Comparison of the coupling constants of this product ($^3J_{7_{ax},8_{ax}}$ = 8.0, $^3J_{8_{ax},9_{ax}}$ = 12.0, $^3J_{9_{ax},10_{eq}}$ = 4.0 Hz) with those of the 9,10-*cis*-phenol adduct (--OC_6H_5 at C-10 of 9) from diol epoxide 1 (28) showed that 9 resulted from cis opening of the oxirane ring of 1 by the 2-amino group of guanine.

A recent publication (22) has assigned the major product from diol epoxide 2 and poly (G) as a trans N^2 substituted guanosine based solely on the nmr and mass spectra of the modified nucleoside. Structural interpretation of the mass spectra at the

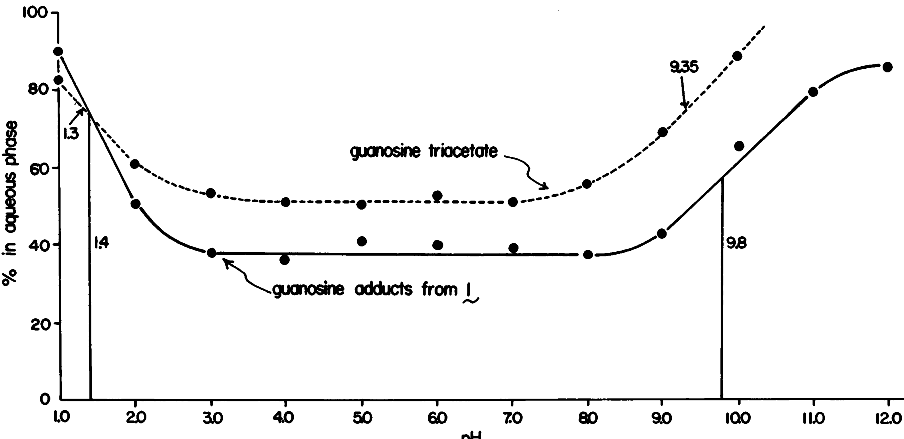

Figure 11. Estimation of pK_a by change in partition coefficient with pH.

Solutions (pH 2.0–9.0) were prepared by mixing 0.05M citric acid pH 1.8, 0.05M K_2HPO_4 pH 7.0, and 0.05M $NaHCO_3$ pH 9.0. Solutions outside the range of pH 2.0–9.0 were obtained by adding HCl or KOH to 0.05M citric acid or $NaHCO_3$. Nucleoside adducts from 1 *or* 2 *were partitioned with 25%* n-*butanol in ethyl acetate. Guanosine triacetate was partitioned with 50%* n-*butanol in ethyl ether. Distribution between the two phases was determined spectrophotometrically as described (*26, 63*).*

Figure 12. Labilization of the glycosidic linkage by N^7 methylation. Diol epoxide 1 *(DEBP) guanosine products were treated with dimethyl sulfate as described in materials and methods.*

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nucleoside level is somewhat difficult since structure 7 (Figure 14) which has a mass 18 units higher than the N^2 substituted analog might be expected to give a similar cleavage pattern after elimination of water. A major argument presented for N^2 substitution was an observed spin-spin coupling between the 2-amino hydrogen and the hydrogen at C-10. A similar coupling between the hydrogen at C-10 and the amino hydrogen at N^7 in 7 would be expected. The formamide signal in 7 would be obscured by the pyrene resonances (64). Although the data presented by these authors (22) does not exclude structure 7, the present tritium release studies with [8-^{3}H]-poly (G) and diol epoxide 2 does. Thus, both diol epoxides 1 and 2 attack the exocyclic 2-amino group of guanosine (22,26).

Assignment of the Absolute Configuration of the Guanosine Adducts from Diol Epoxides 1 and 2. For diol epoxide 1, the minor pair of diastereomers were shown to result from cis opening and the major pair of diastereomers by trans opening of the oxirane ring at C-10. Each of the enantiomers of diol epoxide 1 leads to the formation of a cis and trans adduct. The CD spectra of this cis/trans pair are almost mirror images with opposite signs at their strongest transitions (Figure 8). The cis and trans adducts from a given enantiomer of diol epoxide 1 differ only in their configuration at C-10. Since each enantiomer of diol epoxide 2 also produces a pair of adducts with opposite CD spectra and since the major adduct from each enantiomer of diol epoxide 2 constitute a pair of trans diastereomers (22), it is reasonable to conclude on the basis of the CD spectra that the minor pair of adducts from diol epoxide 2 constitute a pair of cis diastereomers.

Since the absolute configuration of the enantiomeric BP 7,8-dihydrodiols and the four possible corresponding diol epoxides has been assigned (53) and since these diol epoxides have been separately reacted with poly (G), the assignments in Table III are possible. All of these assignments are based on the exciton chirality CD spectrum of the *bis*-(*p*-*N*,*N*-dimethylaminobenzoate) ester of optically active *trans*-7,8-dihydroxy-4,5,7,8,9,10,11,12-octahydro-BP.

Formation of RNA-Nucleoside Adducts from [^{3}H]-BP on Mouse Skin

Once the structures and the chromatographic properties of the adducts which form when diol epoxides 1 and 2 alkylate poly (G) had been established, it became possible to determine whether these products form from BP *in vivo*. Since we have determined the carcinogenicity of BP 7,8-oxide and BP 7,8-dihydrodiol on the skin of C57BL/6J mice, we examined the skin epidermis of this mouse strain for the formation of diol epoxide-nucleoside adducts after topical application of [^{3}H]-BP to the mouse.

Mice were painted with [^{3}H]-BP and the isolated RNA was hydrolyzed to a mixture of nucleosides. After addition of carrier amounts of the eight possible guanosine adducts, the radioactive

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8 9,10-*trans*
9 9,10-*cis*

Figure 13. Structure of the guanosine adducts of diol epoxide 1 after methylation at N^7

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Figure 14. Possible alternative structure of guanosine adducts from trans opening at C-10 of diol epoxide 2 (DEBP)

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nucleosides were purified by Poragel PN chromatography to remove both tetraols and unmodified nucleosides. The radiochemical yield of the products eluted in the guanosine adduct fraction was > 30%. Most of the remaining radioactivity was eluted in the first peak (Figure 6) and is of unknown structure. On this Poragel PN column nucleoside adducts from diol epoxides 1 and 2 and poly (A) are eluted in the tetraol fraction which contained < 5% of the total radioactivity.

When the guanosine-diol epoxide fraction which had been purified by Poragel PN chromatography was examined by HPLC on μC_{18}-Bondapak (Figure 15), radioactive guanosine adducts with chromatographic retention times identical to adduct peaks B, D, F, and G were found. Further confirmation of the nature of the individual radioactive peaks was obtained by determination of their extraction versus pH profile (as illustrated in Figure 11) (26,63). All three radioactive peaks showed a change in partition coefficient in the range of pH 9.0 - 11.0 indicating that each is a N^2 or C^8 substituted guanosine adduct. Adducts at C^8 are presumed not to be present based on the model experiments with poly (G). The pair of adducts B and D arise from diol epoxide 2 and 1, respectively, and provide the first evidence that these diol epoxides are formed and bind to RNA in mouse skin. The formation of both of these diol epoxides was further confirmed by acid hydrolysis of the tritiated nucleoside adducts to radioactive tetraols related to diol epoxides 1 and 2.

The formation of at least three of the eight diastereomeric guanosine derivatives (B and D along with either or both of F and G) provides insight into the metabolism of BP in mouse skin. Peak B is derived from (+)-diol epoxide 2 and guanosine by cis addition at C-10 while peak D is derived from (+)-diol epoxide 1 by trans addition at C-10. (+)-Diol epoxide 2 is formed from (-)-BP 7,8-dihydrodiol, while (+)-diol epoxide 1 is formed from the (+)-enantiomer. Furthermore, the cis/trans counterpart of both peaks B and D (peaks F and G) are known to co-chromatograph with the third radioactive peak. These results imply that the monooxygenases in mouse skin which produce the 9,10-epoxides are highly stereoselective in that predominantly (+)-diol epoxide 1 from (+)-BP 7,8-dihydrodiol and (+)-diol epoxide 2 from (-)-BP 7,8-dihydrodiol form detectable RNA adducts.

Conclusions

We have observed that both diol epoxides 1 and 2 react similarly with poly (G) in 50% acetone/water. Alkylation occurs at phosphate sites (10 - 15%) and at the exocyclic 2-amino group of guanosine. The formation of both cis and trans addition products to the oxirane rings of diol epoxides 1 and 2 suggests that these reactions may proceed at least in part by S_N1 mechanism. This reactivity under neutral conditions is also demonstrated by alkyl-

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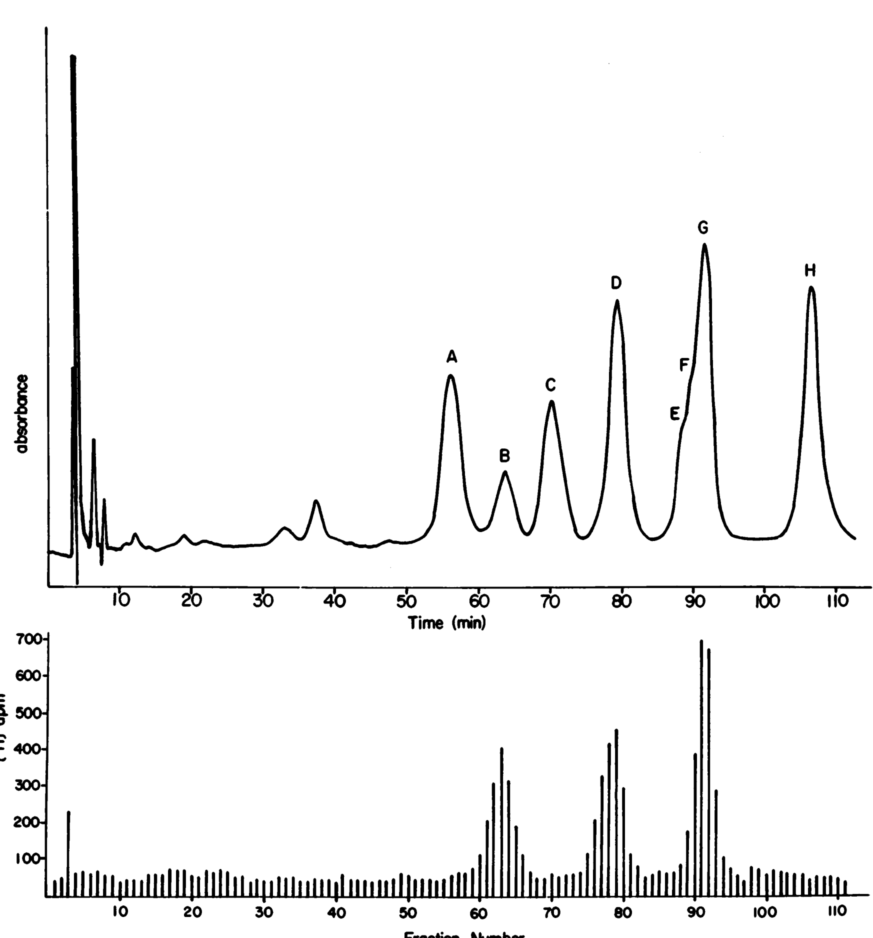

Figure 15. Coinjection of Poragel PN-purified nucleoside adducts from [^{3}H] BP on mouse skin and 2-amino guanosine-diol epoxide adducts from both diol epoxides 1 *and* 2 *on high resolution* μC_{18} *Bondapak. Refer to Figure 7 and Table III for definition of the nucleoside symbols. Cochromatography of the longest retained radioactive peak with uv markers F and G suggests that very little radioactive E was present.*

ation of inorganic phosphate. The relative biological importance of 2-amino substitution and phosphate alkylation can not as yet be estimated. Little is known about the effect of 2-amino substitution by large aromatic groups on factors such as the conformation of the polymer or the fidelity of transcription. Alkylation at phosphate, however, has been suggested to have high biological significance (41,42).

This report describes the structural assignment of the covalently bound guanosine adducts derived from RNA, which are formed when mouse skin is exposed to BP. Both (+)-diol epoxide 1 and (+)-diol epoxide 2 from the (+)- and (-)-enantiomers of BP 7,8-dihydrodiol, respectively, are produced *in vivo* and react with cellular RNA on the 2-amino group of guanosine by cis and trans addition. When bovine bronchial explants were exposed to BP (23) only diol epoxide 2 was observed to form an adduct at the 2-amino group of guanosine. The differences in these two results may be explained by a difference in metabolism of BP between mouse skin and bovine bronchial explants or by the fact that only about half of the radioactivity in the chromatographic region for diol epoxide adducts of guanosine was characterized in the latter study. Whether or not the enantiomers of diol epoxides 1 and 2 are important in the binding of BP to the DNA of mouse skin is presently under study.

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8

Role of Metabolic Activation in Chemical-Induced Tissue Injury

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An important result of metabolism studies in recent years has been the realization that many chemical compounds are metabolized by the liver and various other tissues to potent alkylating and arylating intermediates (1-12). Such studies demonstrate how chemically stable compounds can produce serious tissue lesions in man and experimental animals, including hepatic, renal, and pulmonary necrosis, bone marrow aplasia, neoplasia and other injuries. Although these lesions are rare, such toxic effects are of great clinical concern because they often lead to irreversible failure of the liver, lungs, kidneys or other organs, and subsequent death of the patient.

Many of the initial concepts of metabolic activation were developed during studies of chemical carcinogenesis; the work of the Millers in the United States (1,2) and of Magee and co-workers in England (3) has been especially illuminating. The realization that the enzyme pathways responsible for the conversion of certain chemicals to proximate carcinogens are the same microsomal mixed-function oxygenases that metabolize most drugs and other xenobiotics led to the concept that drug-induced tissue lesions might also be mediated through the covalent binding of reactive metabolites (6-11). The lack of reactivity of most chemically stable compounds and the frequent localization of tissue damage only in those organs or to those animal species having the necessary drug-metabolizing enzymes supported this view. Additionally, these studies frequently demonstrated a role for sulfhydryl-containing compounds, particularly glutathione, in protecting tissues from such toxic reactions.

Most drugs and foreign compounds that enter the body are converted to chemically stable metabolites that are readily excreted into urine and bile, or are expired. Thus, it has become important to distinguish those toxicities that are mediated by chemically reactive metabolites and those reactions due to an exaggerated therapeutic effect or unwanted secondary effect caused by the drug or one of its stable metabolites. The toxicologic activity produced by the latter class of reactions usually can be monitored by

measuring the concentration of the compound and its metabolites in body fluids. However, when the response is tissue damage caused by the covalent binding of chemically reactive metabolites to tissue macromolecules, rarely can a relationship between tissue levels of the metabolite and the severity of the lesion be determined. Indeed, highly reactive metabolites may exist for only a few seconds or less and will therefore never accummulate in body fluids.

Parameters for studying reactive metabolites. How then can the formation of such chemically unstable and reactive metabolites be studied? Based on studies where an animal model has been developed for a particular chemical-induced tissue lesion, a relationship can often be made between the severity of the tissue lesion and the amount of metabolite that is covalently bound to the damaged tissue. That is, covalent binding of the reactive metabolite can be used as an index of formation of the metabolite. Furthermore, this parameter might well be the most reliable estimate of the availability of the metabolite in situ for causing tissue damage, since much of the metabolite often decomposes or is further metabolized before it can be isolated in body fluids. Thus, one approach to the problem is to determine whether radiolabeled drugs administered to animals over a wide dose range are covalently bound to macromolecules in target tissues that subsequently become necrotic. Pretreatment of animals with inducers of drug metabolism, such as phenobarbital, or with inhibitors of drug metabolism, such as piperonyl butoxide, cobalt chloride, or α-naphthylisothiocyanate, similarly should alter the rate of metabolism of toxin, the extent of covalent binding of reactive metabolite, and the severity of tissue injury.

In conjunction with these studies in animals, experiments can be performed in vitro with microsomal enzymes isolated from the target organ tissue. Covalent binding of reactive metabolites may be one useful index of product formation when various additions or deletions from the system are made, or when animals are pretreated with various enzyme inducers and inhibitors. Another useful index of reactive product formation in this system is the trapping of electrophilic intermediates with alternate nucleophiles such as cysteine or glutathione. Structural elucidation of such intermediates may often provide insight into the structure of the initial reactive metabolite. Ultimately, isolation and structure elucidation of the radiolabeled material bound to the tissue macromolecules (RNA, DNA, protein) can be carried out.

The approach described has been used to implicate toxic metabolites as mediators of the toxicities caused by several drugs. Hepatic necrosis has been associated with the use of hydrazides isoniazid (I), a tuberculostatic agent, and iproniazid (II), an antidepressant. Both hepatic and renal injury are associated with the use of high doses of two substituted aminophenol analgesics, acetaminophen (III) and phenacetin (IV). The furan-containing diuretic agent, furosemide (V), and the thiophene-containing

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antibiotic, cephaloridine (VI), are associated with renal injury in man. Ipomeanol (VII), a furan-containing derivative produced by moldy sweet potatoes, is an example of a chemical toxin which produces pulmonary lesions via reactive metabolite formation. These and other experimental studies with model compounds will be presented to illustrate the concepts which underlie the role of metabolic activation in chemical-induced tissue injury and the parameters used to establish these concepts.

Hydrazines and Hydrazides

Isoniazid. A good example of toxic drug reactions caused by metabolic activation is isoniazid-induced liver injury. This drug provides a unique opportunity to show how a study can be pursued from a clinically manifest tissue lesion to the proposal of a rational chemical mechanism for the toxicity.

Clinical findings. Three clinical studies (13-15) provided evidence that metabolic activation was involved in the serious hepatitis caused by isoniazid when this drug was administered in therapeutic doses. First was a prospective study carried out in 1972 (13). SGOT and serum bilirubin concentrations were examined monthly in 250 patients receiving isoniazid for one year. These biochemical indices indicated that isoniazid was hepatotoxic in a large proportion of individuals but most adapted to the insult and recovered rather than developing severe hepatitis. Measurement of plasma concentrations of isoniazid in these patients, failed to show a correlation between plasma levels of isoniazid and liver injury. In this study, no anti-isoniazid antibodies were found and no correlation was seen between hepatic injury and antinuclear antibodies measured at the end of the study.

The seoncd study was a retrospective analysis of 114 patients with isoniazid-related hepatitis (14). Some of the important findings were that: 1) isoniazid-related liver injury was clinically indistinguishable biochemically and morphologically from iproniazid-induced liver damage or from other causes of acute hepatocellular injury such as viral hepatitis; 2) no clinical evidence such as rash, fever, arthralgias or eosinophilia was found for hypersensitivity mechanism; 3) about 30% of the patients with hepatic reactions were residents of Honolulu and of Oriental ancestry; on genetic basis, 90% or more of these patients would be expected to be rapid acetylators of isoniazid in contrast to black and white populations in whom 45% are rapid acetylators (16).

In the third study (15), 21 non-Oriental patients who had recovered from isoniazid hepatitis were genetically phenotyped as rapid or slow acetylators of isoniazid using the sulfamethazine method. Eighty-six percent of them displayed the rapid acetylator phenotype for isoniazid metabolism.

Metabolism studies in man. Based on these clinical findings, we re-examined the metabolism of isoniazid and identified the

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metabolites by co-chromatography, reverse isotope dilution with synthesized standards and by mass spectral analysis (15). Tritium-ring-labeled isoniazid and acetylisoniazid, the major primary metabolite of isoniazid, were administered to human volunteers in single 300 mg doses and urinary metabolites were collected for 24 hrs. As shown in Figure 2, about 55% of a dose of acetylisoniazid was metabolized by hydrolysis to isonicotinic acid and free acetylhydrazine regardless of genetic phenotype of the patients for acetylating isoniazid. In contrast, pattern of metabolites after administration of isoniazid was very dependent upon the rate at which isoniazid was acetylated. On the basis of the relative amounts of acetylisoniazid and isonicotinic acid excreted into the urine, we calculated that almost all of the isonicotinic acid was formed by way of acetylisoniazid. We also calculated that patients who were fast metabolizers of isoniazid converted about 94% of an isoniazid dose to acetylisoniazid; only 2.8% of the drug was excreted unchanged in the urine and 3.6% as hydrazone conjugates. Slow acetylators, on the other hand, excreted almost 37% of the drug in the urine either free or as a hydrazone. Thus, only 63% was converted to acetylisoniazid and subsequently to isonicotinic acid and acetylhydrazine. We concluded, therefore, that fast acetylators are exposed to much more acetylisoniazid and acetylhydrazine than are slow acetylators.

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Hepatic necrosis in animals. Acetylisoniazid and isoniazid were given to rats, mice and hamsters to see if they could produce hepatic necrosis (17,18). These hydrazines were given in a dose-response manner to several hundred animals. Isoniazid did not cause necrosis in any of the animals. However, acetylisoniazid produced occasional single cell necrosis in rats and mice. Moreover, as shown in Table I, pretreatment of rats with phenobarbital, which is known to increase drug metabolizing enzymes, greatly potentiated the necrosis. The liver damage was prevented by pretreatment of rats with cobalt chloride, which inhibits synthesis of cytochrome P-450 metabolizing enzymes. Similarly when hydrolysis of acetylisoniazid was inhibited by pretreatment of rats with bis-para-nitrophenyl phosphate (BNPP), the necrosis was prevented.

The effect on the liver of the hydrolysis product, acetylhydrazine, was therefore examined. This hydrazine is a very potent hepatotoxin which produces hepatic necrosis in phenobarbital-pretreated rats after single doses of 10 mg/kg. The necrosis was potentiated by pretreatment with phenobarbital and prevented by pretreatment with cobalt chloride (Table I). However, BNPP, which inhibited the hydrolysis of acetylisoniazid and prevented the necrosis, had no effect on necrosis produced by acetylhydrazine. Thus, the metabolic activation of the liberated acetylhydrazine moiety of acetylisoniazid to a toxic metabolite satisfactorily accounts for the hepatic necrosis produced by isoniazid.

Subsequently, isoniazid itself was shown to produce acute hepatic necrosis in phenobarbital-treated rats. The proportion of the isoniazid that is acetylated in rats decreases markedly

(I) (II) (III) (IV) (V) (VI) (VII)

Figure 1. Structures of the compounds discussed in the text

DRUG	PATIENTS ACETYLATION RATE (N)	% OF DOSE INH	INH HYDRAZONES	AcINH	INA DERIVATIVES	ESTIMATED ACETYL HYDRAZINE	ESTIMATED HYDRAZINE
AcINH	FAST (2)	---	---	54.9±2.2	45.1±2.7	45.1±2.7	---
AcINH	SLOW (3)	---	---	53.8±1.2	46.2±1.1	46.2±1.1	---
INH	FAST (3)	2.8±0.4	3.6±0.4	49.2±1.9	44.4±3.9	41.0±3.8	3.4±0.1
INH	SLOW (4)	10.9±0.8	26.5±4.8	32.1±1.2	30.5±3.5	26.8±3.3	3.7±0.2

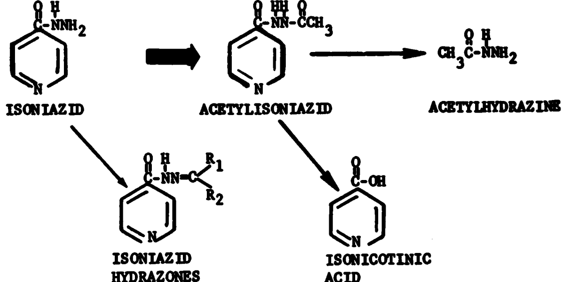

Figure 2. Twenty-four hour urinary excretion of metabolites after administration of 300 mg of acetylisoniazid-^{3}H-ring-labeled (AcINH) or isoniazid-^{3}H-ring-labeled (INH) to male volunteers (See *Ref.* 15)

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Table I

ACUTE HEPATIC NECROSIS IN RATS PRODUCED BY ISONIAZID (INH), ACETYLISONIAZID (AcINH), ACETYLHYDRAZINE (AcHz), IPRONIAZID (IpINH), AND ISOPROPYLHYDRAZINE (IpHz)

Treatments	INH 100 mg/kg*	AcINH 200 mg/kg	AcHZ 20 mg/kg	IpINH 200 mg/kg	IpHz 20 mg/kg
None	0	0 or +	0 or +	+	+
Phenobarbital	+	++	+++	+++	++++
Phenobarbital + $CoCl_2$	0	0	0	0 or +	0 or +
Phenobarbital + BNPP	0	0	+++	0 or +	++++

*Administerd every hour for 6 hours.

+$CoCl_2$ = cobalt chloride.

‡BNPP = bis-para-nitrophenyl phosphate.

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above 100 mg/kg indicating a saturable mechanism. Thus, a single large dose does not cause liver necrosis, but the administration of isoniazid in six single doses of 100 mg/kg per hour caused acute hepatic necrosis (Table I).

Covalent binding studies in vivo. As further support for the hypothesis that acetylisoniazid is converted in the body to a chemically reactive form via activation of acetylhydrazine, ^{14}C-acetylisoniazid radiolabeled in the acetyl moiety and ^{14}C-acetylhydrazine were given to rats and evidence for covalent binding to tissue macromolecules was sought (18,19). A large amount of covalent binding was found upon digestion of the proteins in the liver, the target organ for toxicity, but little was found in other tissues. This binding was proportional to dose, was increased by pretreatment with phenobarbital and was markedly decreased by pretreatment with cobalt chloride (Table II). However, no covalently bound radiolabeled material was found when acetylisoniazid radiolabeled in the pyridine ring was administered. Thus, the reactive metabolite came only from the acetylhydrazine moiety. BNPP, which blocks the hydrolysis of acetylisoniazid, decreased the covalent binding of ^{14}C-acetylisoniazid but not that of ^{14}C-acetylhydrazine, paralleling the effect of BNPP on the hepatic necrosis.

Covalent binding studies in vitro. Based on the effects of mixed function oxygenase inducers and inhibitors on the hepatic necrosis and covalent binding found in animals, experiments were carried out using liver microsomes in vitro to determine the enzyme requirements for the binding reaction. The results of experiments with acetylhydrazine and rat liver microsomes under various conditions (Table III) showed that a substantial amount of covalent binding occurred at 37°C in the presence of liver microsomes, air and NADPH. The binding was almost abolished by lack of NADPH, heat denaturation of the enzymes, or lack of oxygen. A carbon monoxide: oxygen atmosphere, SKF-525A, piperonyl butoxide pretreatment, or an antibody against NADPH cytochrome c reductase inhibited covalent binding, thereby indicating involvement of a cytochrome P-450 mixed function oxygenase. Furthermore, experiments with hepatic microsomes prepared immediately following the traumatic death of a healthy young adult male demonstrate that the activation system is present in human tissues (19, Table III).

Glutathione and cysteine, naturally occurring sulfhydryl compounds, substantially decreased covalent binding in vitro by formation of the adducts, S-acetylglutathione and N-acetylcysteine. The work of Smith and Gorin (21), which showed that S-acetylcysteine rearranges rapidly at neutral pH values to the thermodynamically more stable N-acetylcysteine, suggests that the initial product might have been S-acetylcysteine which subsequently rearranged to the observed product, N-acetlycysteine. Both N-acetylcysteine and S-acetylglutathione were isolated from

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Table II

EFFECT OF TREATMENTS ON IN VIVO HEPATIC COVALENT BINDING OF ISONIAZID-^{3}H-RING-LABELED (INH),* ACETYLISONIAZID-^{3}H-RING LABELED (AcINH)*, ACETYLISONIAZID-^{14}C-ACETYL-LABELED (AcINH), ACETYL-HYDRAZINE-^{14}C-ACETYL-LABELED (AcHz), IPRONIAZID-^{3}H-RING-LABELED (IpINH), IPRONIAZID-2-^{3}H-ISOPROPYL-LABELED (IpINH), and ISOPROPYLHYDRAZINE-2-^{3}H-ISOPROPYL-LABELED (IpHz) IN RATS.

Results are expressed as means ± standard errors of 3 separate experiments using 3 animals in each experiment.

Treatment	AcINH 200 mg/kg	AcHz 20 mg/kg	IpINH 200 mg/kg	IpINH 200 mg/kg	IpHz 20 mg/kg
	Covalent Binding nmole/mg protein (6 hr after dose)			Covalent Binding nmole/mg protein (6 hr after dose)	
None	0.20 ± .021	0.15 ± .012	0.09 ± .015	0.28 ± .029	0.35 ± .023
Pb**	0.31 ± .021	0.19 ± .012	0.10 ± .015	0.53 ± .038	0.44 ± .038
Pb + $CoCl_2$	0.15 ± .039	0.09 ± .008	-	0.18 ± .017	0.22 ± .029
Pb + BNPP‡	0.11 ± .033	0.23 ± .035	-	0.17 ± .019	0.32 ± .025

*Covalent binding for these two compounds was < 0.05 nmole/mg protein for all treatments.

‡Pb + $CoCl_2$ = phenobarbital + cobalt chloride

‡Pb + BNPP = phenobarbital + bis-para-nitrophenyl phosphate

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Table III

COVALENT BINDING *IN VITRO* OF ACETYL-(^{14}C)-HYDRAZINE (^{14}C-AcHz) AND ISOPROPYL-(2-^{3}H)-HYDRAZINE (^{3}H-IpHz) TO RAT LIVER MICROSOMES

Conditions	^{14}C-AcHz (1 mM)	^{3}H-IpHz (0.1 mM)
	% of Control**	
A. Control* (air atmosphere)	100%	100%
Boiled microsomes	13%	12%
-Cofactor (NADPH generating system)	6%	10%
+NADH (-NADPH generating system)	7%	7%
100% N_2 atmosphere	15%	11%
N_2:O_2 (9:1) atmosphere	92%	97%
CO:O_2 (9:1) atmosphere	37%	48%
+SKF 525-A (0.2 mM)	64%	70%
Piperonyl butoxide+	25%	45%
+GSH (1 mM)	35%	58%
+Cysteine (1 mM)	49%	65%
	nmoles/mg/15 min	
B. Preimmune γ-globulin‡	0.099	0.328
Immune γ-globulin‡ (NADPH-cytochrome c reductase antibody)	0.048	0.159
	Human Microsomes	
C. Control (air atmosphere)	0.16	0.37
-cofactor (NADPH generating system)	0.02	0.03

*Microsomes were prepared from rat liver and human liver, incubated as described in Table IV and covalent binding was determined (19).

**The control binding of AcHz with rat liver microsomes was 0.55 nmoles/mg/15 min and for IpHz was 0.58 nmoles/mg/15 min.

+Administered (0.3 ml) i.p. 30 min prior to sacrificing the animal.

‡Each incubation contained 7 mg of partially purified preimmune or immune γ-globulin per mg microsomal protein, as previously described (20).

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incubation mixtures by gel filtration on Sephadex followed by anion exchange chromatography. The products were then characterized by chemical ionization mass spectrometry (22,23).

Iproniazid. Studies in animals of the metabolism of iproniazid (II), an antidepressant drug removed from clinical use because of a high incidence of hepatitis similar to that of isoniazid revealed that iproniazid also required enzymatic hydrolysis to produce the hepatic lesion (Table I). Specific radiolabeling and covalent binding studies showed that isopropylhydrazine was released by hydrolysis and then oxidatively activated in vitro to a potent hepatotoxin (19, Table II).

Metabolic activation of isopropylhydrazine, the hepatotoxic metabolite of ipronazid, to a reactive intermediate showed enzyme requirements virtually identical to those for the activation of acetylhydrazine (Table III). Thus, a cytochrome P-450 oxygenase mediated the covalent binding of isopropylhydrazine to tissue protein. Trapping experiments with cysteine and glutathione showed that S-isopropylcysteine and S-isopropylglutathione were formed (23).

The activating enzyme system could be assessed kinetically using covalent binding of radiolabeled metabolite as an index of reactive product formation. A double-reciprocal plot of the enzyme-dependent binding of acetylhydrazine to microsomal proteins (Figure 3A) shows that the reaction rate is markedly increased by phenobarbital pretreatment, which potentiated the hepatic necrosis and binding in vivo, whereas it is decreased by pretreatment of the animals with cobalt chloride, which blocked the hepatic necrosis and binding in vivo.

The same effects were found for the binding reaction of isopropylhydrazine to rat liver microsomes, except that the K_m for binding was 1/10 that for acetylhydrazine (Figure 3B). This may account for the greater hepatotoxicity observed with isopropylhydrazine when compared to that of acetylhydrazine. In additon, the evolution of propane from microsomal reactions was determined by gas chromatography and gas chromatography-mass spectrometry.As shown in Table IV, phenobarbital increased both covalent binding and propane formation, whereas cobalt chloride decreased both.

Double-isotope experiments with acetylisoniazid-acetylhydrazine and iproniazid-isopropylhydrazine. In order to study the metabolic activation process for the covalent binding of acetylisoniazid and acetylhydrazine in more detail, we administered to rats a mixture of acetylisoniazid and a mixture of acetylhydrazine labeled with tritium and carbon-14 in the acetyl moiety. The $^3H/^{14}C$ ratio of the covalently bound metabolite from either acetylisoniazid or acetylhydrazine was almost identical to the $^3H/^{14}C$ ratio of the administered mixture (19, Table V). This indicated that the entire acetyl group was bound. Moreover, incubation of the ^3H- and ^{14}C-acetylhydrazine with rat liver microsomes in vitro gave the same results (19, Table V).

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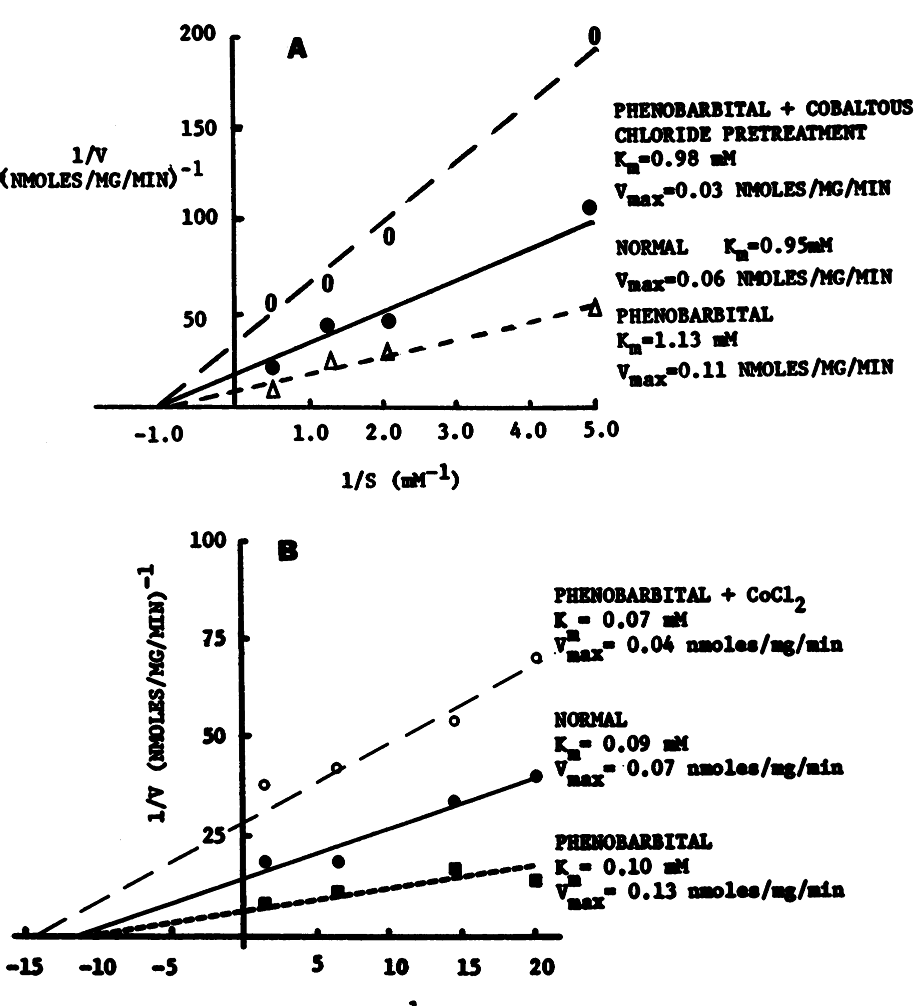

Figure 3. Lineweaver–Burk plots of mixed function oxidase-dependent covalent binding of acetylhydrazine (A) and isopropylhydrazine (B) to rat microsomal protein in vitro. For each incubation, rat microsomes were prepared, incubated under air with either ^{14}C-acetylhydrazine (A) or isopropyl-2-(^{3}H)-hydrazine (B) and a NADPH-generating system, and covalent binding was determined.

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TABLE IV
CORRELATION OF PROPANE EVOLUTION WITH *IN VITRO* COVALENT BINDING OF ISOPROPYL-(2-^{3}H)-HYDRAZINE TO HEPATIC MICROSOMES

Ice-cold incubation mixtures (3 ml) contained rat liver microsomal protein (2 mg/ml) isolated from rats pretreated as indicated; phosphate buffer, pH 7.4, 83 mM; isopropyl-(2-^{3}H)-hydrazine, 0.1 mM; and a NADPH-generating system (NADP, 0.64 mM; glucose-6-phosphate, 15.5 mM; glucose-6-phosphate dehydrogenase, 2U/ml; $MgCl_2$, 10 mM). Reactions were incubated under air in septum-sealed incubation vessels for 15 min with shaking (Dubnoff shaker incubator) at 37°C and covalent binding determined (19). The head-space gases were analyzed by GLC as described in Ref. 19; the propane effluent was trapped in Aquasol scintillant cooled in dry ice-acetone, and radioactivity was counted by scintillation spectrometry. Results are expressed as means ± standard deviations. Numbers in parentheses are number of determinations.

Treatment	*In vitro* Covalent Binding (nmoles/mg protein/15 min)	Propane evolved (% of total radioactivity in 15 min)
None	0.58 ± 0.051 (9)	13.0% ± 1.00 (9)
Phenobarbital (75 mg/kg i.p. x 4 days)	1.06 ± 0.059 (6)	19.5% ± 0.75 (6)*
Phenobarbital (75 mg/kg i.p. x 4 days) + cobalt chloride (30 mg/kg s.c. 12 hourly x 4 doses)	0.33 ± 0.006 (6)	9.4% ± 0.81 (6)*

*P ≤ 0.05 when compared to respective control values as determined by Student's t test.

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Table V

RATIOS ($^3H/^{14}C$) RADIOLABEL BOUND TO HEPATIC PROTEIN VERSUS THAT IN INITIAL SUBSTRATE MIXTURES

Mixtures of ^{14}C-carbonyl- and ^3H-methyl-labeled acetyl-isoniazid (AcINH; 200 mg/kg; sp. act. ^{14}C, 0.15 mCi/mmole; ^3H, 0.53 mCi/mmole) and similar mixtures of acetylhydrazine (AcHz, 20 mg/kg; sp. act. ^{14}C, 0.36 mCi/mmole; ^3H, 1.01 mCi/mmole) were administered to male Fischer rats. In addition, mixtures of specifically labeled isopropyl-(2-^{14}C)- and isopropyl-(2-^3H)-labeled iproniazid (IpINH, 200 mg/kg; sp. act ^{14}C, 0.50 mCi/mmole; sp. act. ^3H, 1.43 mCi/mmole) and isopropylhydrazine (IpHz, 20 mg/kg; sp. act. ^{14}C, 0.30 mCi/mmole; sp. act. ^3H, 0.87 mCi/mmole) were administered to Fischer rats. In other experiments, mixtures of ^3H- and ^{14}C-acetylhydrazine (1 mM) and isopropyl-(2-^3H)- and isopropyl-(2-^{14}C)- hydrazine (0.1 mM) were incubated in air with an NADPH-generating system and with microsomes isolated from Fischer rat liver. Covalent binding of radiolabel to hepatic tissue protein was determined by methods previously described (19) and found to be 0.20 nmoles/mg (*in vivo*, AcHz), 0.28 nmoles/mg (*in vitro*, IpHz). Values are reported as $^3H/^{14}C$ ratios of the covalently bound radiolabel, as determined by the channels-ratio method using integral counting, divided by the $^3H/^{14}C$ ratio of the initial substrate mixture as determined by the same method. Results are expressed as means ± standard errors of 4 such determinations.

Conditions	Substrate			
	AcINH	AcHz	IpINH	IpHz
In vivo (6 hr after dosing)	0.90 ± .055	0.92±0.021	0.92±.032	0.96±0.060
In vitro (15 min incubations)	-	0.94±0.012	-	0.98±0.022

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Liver microsomes and NADPH were also incubated with cysteine and approximately equimolar amounts of acetyl- and trideuteroacetylhydrazine (22). N-acetylcysteine was isolated from incubation mixtures containing NADPH, oxygen, cysteine and acetylhydrazine. Chemical ionization mass spectrometry showed quasimolecular ions (QM^+) at m/e 164 and 167 for the non- and trideuterated N-acetylcysteine. These ions were monitored and found to have the same H/D ratio as the quasimolecular ions of the acetylhydrazine substrate mixture (m/e 75 and 78, Figure 4). This study eliminated ketene as the reactive intermediate.

Similar experiments with iproniazid and isopropylhydrazine, labeled with tritium and carbon-14 in the methine carbon of the isopropyl group, showed that equivalent amounts of tritium and carbon-14 were bound both *in vivo* and *in vitro*, demonstrating that methine hydrogen was retained and therefore eliminating acetone as the intermediate in the binding reaction (19).

This was confirmed by a twin-ion study using specifically C-2 deuterated isopropylhydrazine. The reactive metabolite was trapped from microsomal incubations *in vitro* with cysteine. Mass spectral analysis of the isolated cysteine derivative showed that S-isopropylcysteine was formed and that no deuterium was lost from the isopropyl group (23).

Mechanistic implications for the metabolic activation of toxic metabolites of isoniazid and iproniazid. From the results *in vivo* showing correlations between tissue necrosis and covalent binding, studies *in vitro* showing a microsomal P-450 oxygenase requirement, and trapping experiments with cysteine and glutathione, we propose the following reaction scheme (Figure 5) for formation of toxic metabolites from isoniazid and iproniazid.

Isoniazid is acetylated to its major metabolite acetylisoniazid. In man, rapid acetylators convert at least 35% more isoniazid to acetylisoniazid than slow acetylators. Acetylisoniazid is then efficiently hydrolyzed to isonicotinic acid and acetylhydrazine. Acetylhydrazine is further metabolized by a P-450 oxygenase, possibly to a N-hydroxy hydrazine. This intermediate would probably dehydrate to acetyldiazene which could be the electrophilic acylating species. However, mono-substituted diazenes are known to fragment in the presence of oxygen most likely to radicals (24) and this could be the toxic intermediate. Ketene has been eliminated as the reactive acylating species of acetylhydrazine by chemical ionization mass spectral twin-ion study with trideutero-acetylhydrazine, which indicated that the entire acetyl group was bound (22). By mechanisms that are not understood the reactive metabolite initiates processes that lead to hepatic necrosis.

Iproniazid is hydrolyzed to isonicotinic acid and isopropylhydrazine. Isopropylhydrazine is then further oxidized to a reactive alkylating agent. Since the evolution of propane parallels covalent binding, we suspect the two reactions derive from common

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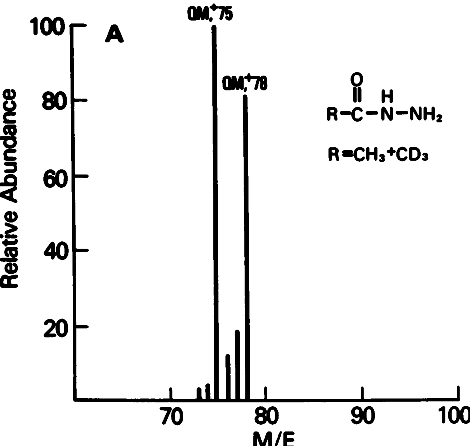

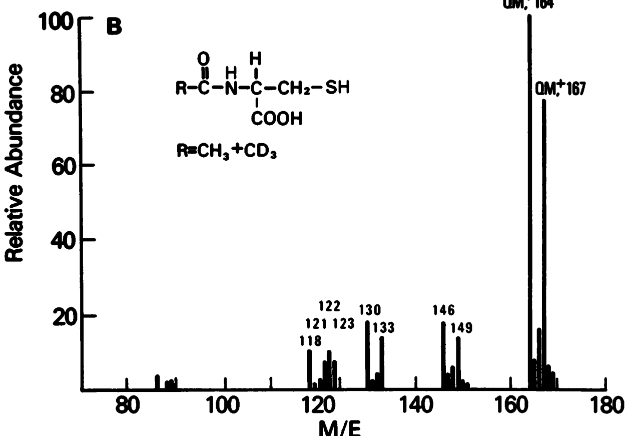

Figure 4. Chemical ionization mass spectra (isobutane reactant gas) of a sample of the substrate mixture of acetyl- and trideuteroacetylhydrazine (A) and of the cysteine adduct isolated from a microsomal incubation containing the substrate mixture, NADPH and cysteine (B) (See *Ref.* 22)

intermediate. Furthermore, a twin-ion study with specifically C-2 deuterated isopropylhydrazine, similar to the study carried out with trideuteroacetylhydrazine, indicated that the entire isopropyl group was retained in the bound metabolite. A reaction scheme (Figure 5) compatible with these results is the formation of the isopropyl radical or cation from isopropyldiazene. These reactive alkylating agents then covalently bind to tissue macromolecules.

Whatever the intermediate may be, it is clear that oxidative activation of these hydrazines by microsomal enzymes mediates liver necrosis in animals. Since these enzymes are present in human liver tissue, these intermediates probably cause the serious and occasionally lethal hepatitis seen with isoniazid and iproniazid therapy in man.

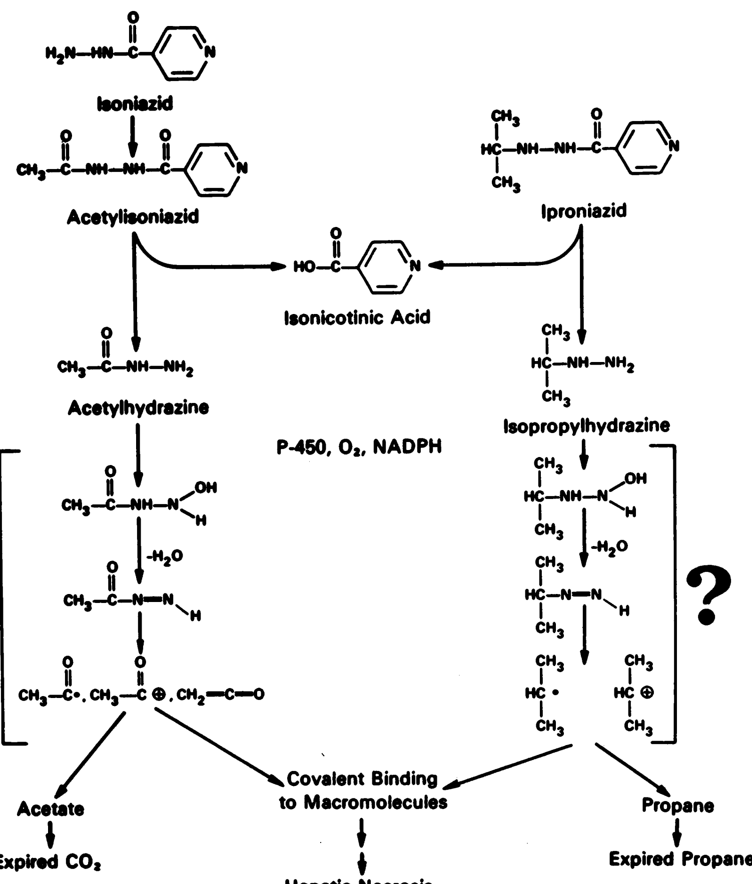

Figure 5. Proposed metabolic activation pathways for isoniazid, acetylisoniazid, and isopropylisoniazid (iproniazid)

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Acetaminophen

Studies in vivo in man and laboratory animals. Acetaminophen (p-hydroxyacetanilide, III) is a commonly used mild analgesic which is apparently quite safe when taken in normal therapeutic doses. However, large overdoses cause life-threatening liver lesions in man (25,26), rats (27,28), mice (28), and hamsters (29). Prior treatment of animals with inducers of drug metabolism, such as phenobarbital or 3-methylcholanthrene, greatly potentiates the severity of the necrosis (28,30). In contrast, pretreatments with inhibitors of drug metabolism, such as piperonyl butoxide, cobalt chloride, or α-naphthylisothiocyanate, prevent the necrosis (28,30). A lack of correlation between acetaminophen tissue levels and acetaminophen-induced hepatic necrosis indicates that a toxic metabolite rather than acetaminophen itself causes the hepatic tissue injury.

Acetaminophen radiolabeled with tritium or with carbon-14 was given to normal mice and mice pretreated with compounds that altered acetaminophen-induced hepatic necrosis. The animals were killed at various times and the livers examined for covalently bound metabolites of acetaminophen. Autoradiograms showed covalent binding of acetaminophen preferentially in the necrotic centrilobular area of the liver, i.e., there was a direct correlation between the two measurable parameters tissue necrosis and covalent binding (31). Pretreatment with an inducer of microsomal metabolism, phenobarbital, increased binding, whereas pretreatments with different inhibitors of metabolism decreased binding. Thus, the effect of treatment on covalent binding correlated directly with treatment effects on hepatic necrosis. Evidence for the covalent nature of the binding was obtained by digestion of solvent-extracted liver protein with protease and isolation of the radiolabel bound to amino acid and peptide fragments. These studies indicated that acetaminophen was converted by microsomal enzymes, to a reactive arylating agent which covalently bound to macromolecules in the target tissue for damage, the liver.

Concept of a dose-threshold for toxicity. Because of the striking correlation between the severity of hepatotoxicity and the extent of covalent binding by the arylating metabolite of acetaminophen, it was surprising that significant binding did not occur until over 60% of the drug had been eliminated from the liver. Glutathione is depleted from the liver of animals receiving acetaminophen because it combines with a minor metabolite of the drug and forms a readily excreted mercapturic acid (4,30,32,33). Thus the possibility arises that the arylating metabolite of acetaminophen intially is detoxified by reacting preferentially with glutathione (Figure 6). After the major routes of acetaminophen metabolism (sulfation and glucuronidation pathways) become saturated, and after the liver is depleted of glutathione, the reactive metabolite can combine with liver macromolecules and by undefined mechanisms cause cell death.

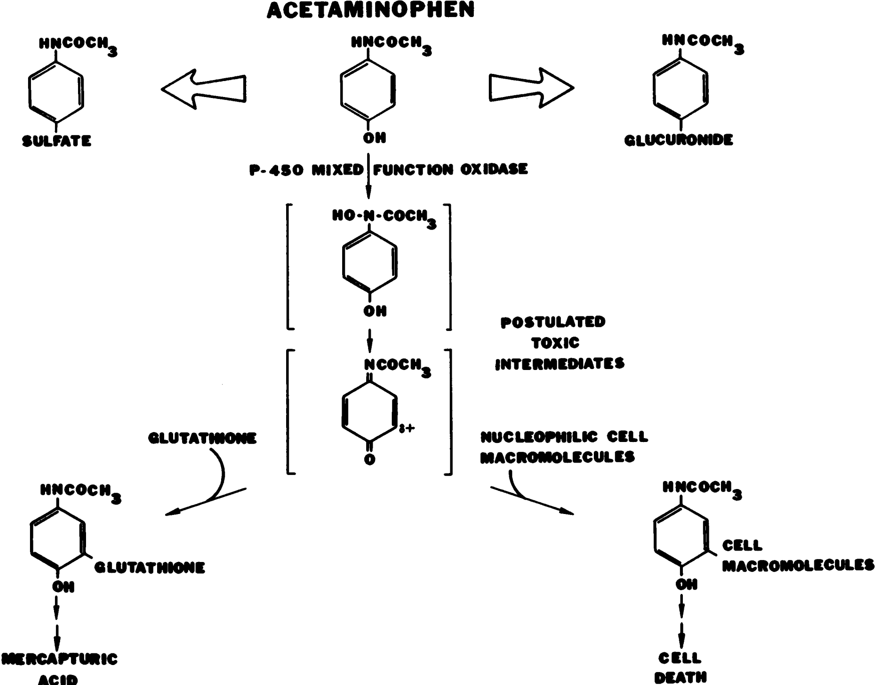

Figure 6. Pathways of acetaminophen metabolism

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In support of this view, covalent binding and liver necrosis occurred only after doses of acetaminophen sufficiently large to exceed the availability of glutathione for detoxification (Figure 7). Similarly, when glutathione concentrations in the liver were compared with the extent of covalent binding at various times after the administration of acetaminophen, significant binding had occurred only after glutathione was severely depleted (30,32). In accord with this view, prior administration of diethyl maleate, which decreases the glutathione concentration in liver without causing liver necrosis, markedly potentiates the liver damage caused by acetaminophen (30,32), and diets that lower the concentration of glutathione enhance the toxicity as well (34). On the other hand, the administration of the alternate sulfhydryl compounds, cysteine or cysteamine, prevented the liver necrosis (6,32).

Recent studies with acetaminophen have supported the view that a glutathione threshold is operative in man as well as laboratory animals (33,35,36). Therefore, sulfhydryl reagents such as cysteine, cysteamine, dimercaprol, and glutathione itself are being successfully used in the therapy of acetaminophen-overdosed patients (37). This emphasizes the importance of understanding biochemical mechanisms of toxicity before rational approaches to treatment can be made.

Phenacetin

Phenacetin (p-ethoxyacetanilide, IV) has been implicated in renal injury in man (38). Therefore, we considered the possibility that this special type of nephritis, called analgesic nephropathy, was related to metabolic activation. Although no consistently reproducible lesion could be obtained in laboratory animals treated with large doses of phenacetin, liver necrosis was observed, especially in hamsters (39), a species unusually susceptible to acetaminophen-induced hepatic necrosis (29,30). As with acetaminophen, phenacetin-induced liver necrosis in hamsters is potentiated by pretreatment with 3-methylcholanthrene but not by phenobarbital. For example, phenacetin doses of 400 mg/kg produce massive centrilobular necrosis in 3-methylcholanthrene-treated animals. Moreover, the severity of necrosis parallels the magnitude of the covalent binding of radiolabeled phenacetin to hepatic proteins and the depletion of hepatic glutathione (39). Little binding or hepatic necrosis occurs at doses that deplete hepatic glutathione less than 80%. However, considerable binding and necrosis occur at doses that deplete glutathione more than 80%. Pretreatment of hamsters with 3-methylcholanthrene increases depletion of hepatic glutathione, the covalent binding, and the severity of necrosis after phenacetin, whereas pretreatment with cobaltous chloride or piperonyl butoxide decreases them. These findings indicate that glutathione in the liver prevents covalent binding and necrosis by combining with a reactive arylating metabolite of phenacetin.

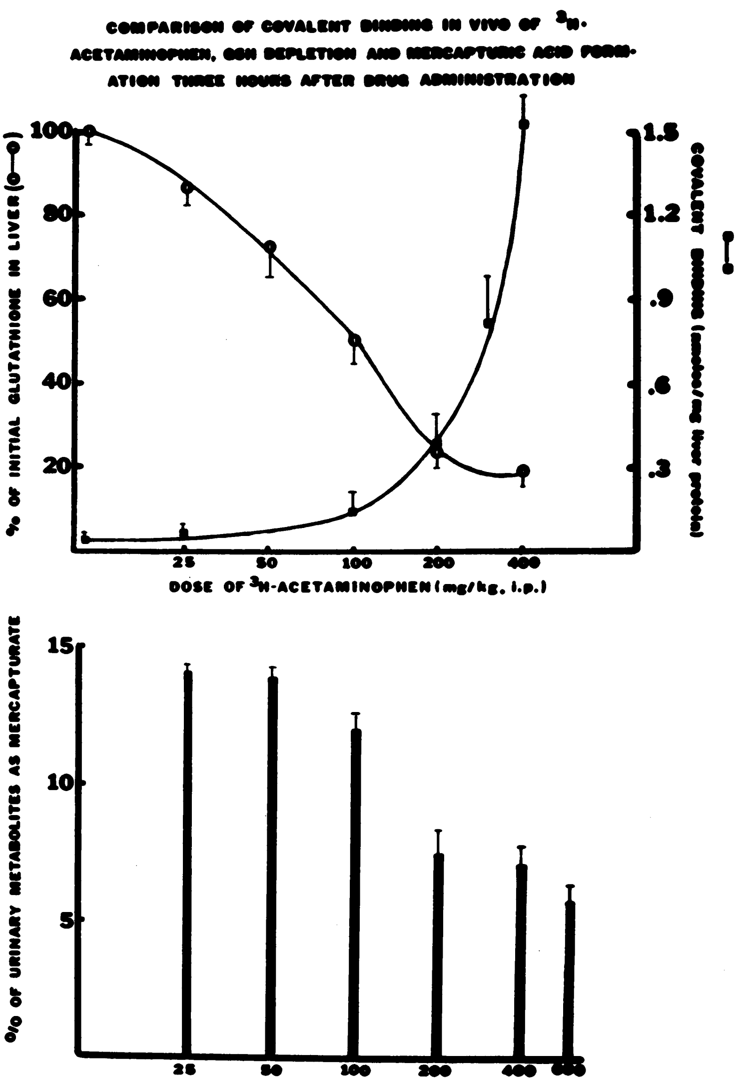

Figure 7. Relationship in vivo between hepatic glutathione concentration, the formation of an acetaminophen-glutathione conjugate (measured in urine as acetaminophen mercapturic acid), and covalent binding of an acetaminophen metabolite to liver proteins (See *Ref.* 33)

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Mechanisitic implications for the metabolic activation of acetaminophen and phenacetin based on studies using hamster microsomal enzymes. Although the identity of the arylating metabolites of acetaminophen and phenacetin are uncertain, the involvement of N-hydroxy derivatives or arene oxides as reactive metabolites has been postulated (30, 40). Experiments in vitro with hamster liver microsomes suggests that the arylating metabolites of acetaminophen and phenacetin are different, at least in this microsomal system (41). The evidence is as follows: 1)The maximum velocity of covalent binding for phenacetin exceeds that for acetaminophen, showing that phenacetin is not first deethylated to acetaminophen which is then activated. 2)Pretreatment of hamsters with 3-methylcholanthrene increases the rate of covalent binding for acetaminophen but decreases the rate of binding for phenacetin. 3) Phenobarbital pretreatment increases the rate of covalent binding for phenacetin without affecting the rate of binding for acetaminophen. 4) When covalent binding was prevented by trapping of the reactive metabolites with glutathione during incubations carried out under atmospheres of oxygen-18, reduction by Raney-nickel of the glutathione conjugates formed from either acetaminophen or phenacetin yielded acetaminophen; however, the acetaminophen conjugate formed during incubation with phenacetin incorporated 50% oxygen-18 into the 4-position, whereas the acetaminophen-glutathione conjugate during incubation with acetaminophen incorporated virtually no oxygen-18.

Mechanisms based on these results are presented in Figure 8. The lack of incorporation of oxygen-18 into the glutathione conjugate derived from acetaminophen is consistent with either an N-hydroxylation or 2,3-epoxidation mechanism. Indirect evidence in vivo supports an N-hydroxylation mechanism for the metabolic activation of acetaminophen. Masking of the amide nitrogen, as in N-methyl-4-hydroxy acetanilide, blocks hepatotoxicity (39). Pretreatment of hamsters with 3-methylcholanthrene, which increases the hepatotoxicity of acetaminophen, correspondingly increases the N-hydroxylation of 4-chloroacetanilide and 2-acetylaminofluorene and the covalent binding of acetaminophen (20, 42, 43) By contrast, phenobarbital pretreatment neither alters the rate of N-hydroxylation of 4-chloroacetanilide nor the covalent binding of acetaminophen (43). It seems likely that if an N-hydroxylated metabolite were formed it would subsequently undergo dehydration to a chemically reactive imidoquinone before arylating tissue macromolecules (Figure 8).

The incorporation of 50% oxygen-18 into the 4-position of the arylating metabolite of phenacetin is perplexing. The strong implication is that the carbon atom in this position (C-4 of the aromatic ring) becomes tetrahedral, binding equivalent oxygen-18, derived from molecular oxygen, and oxygen-16. Mechanisms consistent with this interpretation are presented in Figure 8.

Although these mechanisms are consistent with the results obtained in vitro with phenacetin, they may have little bearing on the situation in vivo. The major metabolite of phenacetin in vivo

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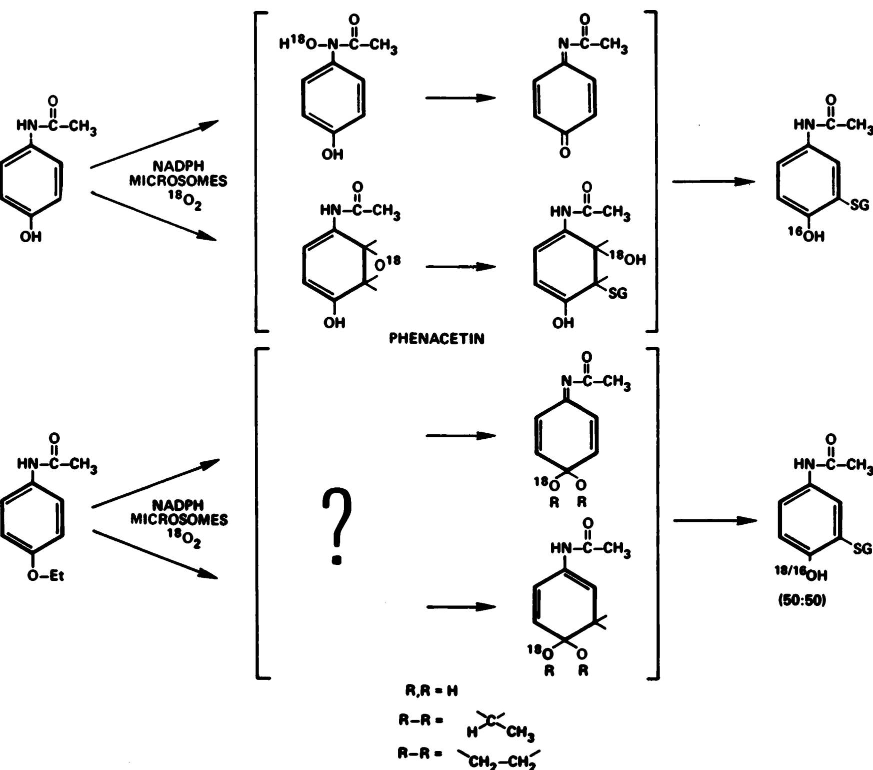

Figure 8. Possible reaction mechanisms for reactive metabolite formation from acetaminophen and phenacetin using hamster liver microsomes

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is acetaminophen and those pretreatments in hamsters which increase hepatic necrosis and covalent binding for acetaminophen increase the hepatic necrosis and covalent binding for phenacetin (39). Additional studies in vivo with phenacetin, specifically deuterated in the α-methylene carbon atom of the 4-ethoxy group, also indicated that deethylation of phenacetin to acetaminophen is at least partially rate-determining for hepatic tissue injury (44, 45).

Renal injury with acetanilides. Reactive intermediates of acetaminophen, phenacetin, and other acetanilides may also mediate the renal injury caused by these compounds. Nery (46) has suggested that the N-hydroxylated derivative of phenacetin is an intermediate for minor urinary metabolites of phenacetin. Calder et al. (47) subsequently examined the nephrotoxicity of N-hydroxyphenacetin, 4-aminophenol, hydroquinone, and p-benzoquinone and found acute renal tubular necrosis in rats. It therefore seemed possible that acetaminophen and phenacetin could be nephrotoxic through their N-hydroxy metabolites and ultimately through the common reactive derivative, N-acetyl-4-benzoimidoquinone.

Both acetaminophen and phenacetin covalently bind to a small extent to Fischer rat kidney and acetaminophen causes renal tubular necrosis (39). Both compounds deplete renal glutathione, with acetaminophen much more potent than phenacetin. Experiments in vitro with rat kidney microsomes show that these compounds can be activated to reactive metabolites. Alternatively, acetaminophen and phenacetin may be N-hydroxylated in the liver and transported to the kidney, possibly as N-O-glucuronides. The glucuronides might then be hydrolyzed under the acidic conditions in the urine or by glucuronidases in the kidney or urine to cause the observed renal injury. However, 3-methylcholanthrene pretreatment increases the hepatic necrosis, but slightly decreases the renal necrosis produced by acetaminophen, suggesting that the acetaminophen activation responsible for renal injury occurs in the kidney itself.

Furans and Thiophenes

Furosemide

Toxicity in vivo of furosemide. Furosemide (V), a frequently used diuretic drug, is contraindicated in pregnancy because of its recognized teratogenic potential (48). The drug also has been reported to potentiate renal injury when used in combination with cephaloridine (49, 50). Furosemide produces massive hepatic necrosis in mice and the necrosis is prevented when metabolism is inhibited by pretreatment of mice with piperonyl butoxide, cobalt chloride and α-naphthylisothiocyanate (51). Covalent binding of the drug to hepatic tissue in mice is also blocked by these pretreatments and occurs a few hours before histologically recognisable necrosis. Thus, the formation of a reactive furosemide metabolite is causally related to the development of furosemide-induced hepatic necrosis.

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As with acetaminophen and phenacetin, the hepatic necrosis produced by furosemide also exhibits a dose-threshold for toxicity. No covalent binding or necrosis occurs until a dose of 100 mg/kg is exceeded. Unlike the dose threshold for acetaminophen and pheneacetin hepatotoxicity, the furosemide threshold is not due to a protective role of glutathione, since furosemide does not deplete hepatic glutathione. Studies of metabolism, distribution, and reversible plasma protein binding of furosemide after toxic and nontoxic doses indicate that the dose-threshold for toxicity results from either saturation of the organic anion binding sites on plasma proteins after toxic doses, or possibly saturation of biliary or renal excretion of the drug (52).

Possible mechanism of metabolic activation. The hepatic injury produced by furosemide apparently results from the metabolic activation of the furan ring, possibly by an epoxidation similar to that proposed in Figure 9. Furosemide, radiolabeled with tritium in its furan moiety, is bound covalently to hepatic microsomes in the presence of oxygen and NADPH to the same extent as furosemide radiolabeled specifically with sulfur-35 in its sulfonamide moiety, demonstrating that the bound metabolite contains both parts of the furosemide molecule. To determine where the binding occurred on the molecule, the metabolite-protein conjugates isolated from the liver were hydrolyzed under mild acid conditions that split furosemide into its methylfuran and sulfamyolanthranilic acid portions. The binding of furan-radiolabeled furosemide to protein was unchanged, whereas the binding of ^{35}S-labeled furosemide was lost. The results suggested that the furan ring was being metabolically activated (51, 52).

Additional studies in vitro reported by Wirth et al. (53) using$_2$ [α-^{3}H] furosemide, [35S] furosemide], [α-^{2}H] furosemide, and [α,α-^{2}H] furosemide indicated that formation of a possible electrophilic imine intermediate was unlikely and that the α-carbon was not a site of metabolic activation. This further implicated the furan ring. Since covalent binding was enhanced by an epoxide hydrase inhibitor and did not occur when tetrahydro [35S] furosemide was used as substrate, the authors speculated that an arene oxide intermediate of the furan moiety was involved in the binding.

4-Ipomeanol

Toxicity in vivo of 4-ipomeanol. Chemicals which reproducibly produce an acute, specific pulmonary toxicity by routes of administration other than inhalation are rare and their mechanisms of action are poorly understood. 4-Ipomeanol (VII), a 3-substituted furan and the major component of "lung edema factor" produced in sweet potatoes (Ipomoea batatas) infected with a common mold, has provided a valuable tool with which to probe chemical-induced lung disease(54).The ingestion of mold-damaged sweet potatoes has been implicated for many years in outbreaks of poisoning in cattle. Affected animals suffer severe and often fatal respiratory distress.

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The same kind of lung damage observed in cattle can be produced in laboratory animals by administration of synthetic 4-ipomeanol (55, 56). The lung is the primary target organ in most species. Pathological changes, such as pleural effusions, intra-alveolar and perivascular edema are apparent within 6-24 hours after administration of the toxin.

Studies were undertaken to determine the possible formation of a chemically reactive metabolite in this pulmonary toxicity. Rats have been used as the experimental species in all experiments described here. Pretreatments of animals with metabolic inhibitors such as pyrazole, piperonyl butoxide, and cobalt chloride all markedly reduced the toxicity of 4-ipomeanol in the lung (57, 58). Phenobarbital, an inducer of mixed function oxygenase activity did not alter the nature of the lung toxicity but significantly increased the LD50 value, possibly by increasing detoxification pathways more than toxic pathways (59). Another type of inducer, 3-methylcholanthrene showed a striking phenomenon when it was used to pretreat rats. Mortality was decreased because of marked decrease in lung damage. In contrast, tissue injury increased dramatically in the liver with appearance of widespread centrilobular necrosis (60). Thus, the target organ for toxicity had switched from the lung to the liver.

Covalent binding in vivo and in vitro. In non-pretreated rats, radioactivity from 14C-4-ipomeanol becomes covalently bound, preferentially to the lungs, after all routes of administration. Pretreatments with mixed-function oxygenase inhibitors, which decreased lung toxicity, caused a parallel decrease in covalent binding to lung tissue *in vivo* and to lung microsomes *in vitro* (57, 58, 61). Phenobarbital pretreatment also decreased the covalent binding of toxin to both lung and liver tissue *in vivo*. However, the maximal rate of binding to liver microsomes *in vitro* was increased whereas no change occurred in binding to lung microsomes.

The alteration of target organ specificity for tissue damage observed with 3-methylcholanthrene pretreatment was paralleled by similar alteration of covalent binding of radiolabeled 4-ipomeanol *in vivo* (60). The level of covalently bound toxin was markedly elevated in livers of 3-methylcholanthrene-induced rats, whereas binding to lung was significantly reduced. The rate of binding to liver microsomes *in vitro* was also markedly increased, whereas the rate of binding to lung microsomes was unchanged (60).

An important conclusion can be drawn from the experiments in 3-methylcholanthrene pretreated animals. The toxic metabolite of 4-ipomeanol is so reactive that little, if any, of it escapes the organ in which it is formed. It therefore appears that in normal animals the specific lung toxicity produced by 4-ipomeanol results primarily from pulmonary metabolism of the agent; the liver is not a significant source of the reactive metabolite that binds to and damages the lungs. The increase in hepatic toxicity in 3-methylcholanthrene pretreated animals is due to an increased hepatic metabolism of 4-ipomeanol; the reduction in pulmonary toxicity

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probably is due to an elevated rate of hepatic clearance of toxin.

Nature of the chemically reactive metabolite of 4-ipomeanol. Studies in vivo and in vitro indicate that the reactive metabolite formed by mixed function oxygenase-catalyzed metabolism of 4-ipomeanol is a highly electrophilic species (57, 58). Addition of the nucleophilic tripeptide, glutathione, markedly inhibited covalent binding of 4-ipomeanol in vitro, presumably by acting as an alternate nucleophile. Depletion of endogenous glutathione in vivo by diethylmaleate pretreatment significantly increased toxicity and covalent binding of 4-ipomeanol in vivo.

Analogs of 4-ipomeanol in which the furan moiety was replaced by phenyl or methyl substituents were not metabolized to toxic electrophiles in vivo or in vitro (58). Thus, the furan ring appears to be essential for the observed toxicity and covalent binding. Based on these results and those found for the metabolic activation of furosemide, Figure 10 reveals a possible scheme for furan activation. Since furan has less aromaticity than benzene, it is not unlikely that this heteroaromatic nucleus could form an epoxide. This epoxide would probably be quite reactive, and could yield other electrophiles by spontaneous rearrangement or ring scission reactions. This arene oxide could also be deactivated by an epoxide hydrase, glutathione, or a glutathione transferase. Relative activities of the various pathways in the various tissues would modulate susceptibility to the toxin.

Hepatotoxicity, renal toxicity, and pulmonary toxicity are also caused by other furan compounds, some of which show a glutathione threshold, and others which show no such threshold (51). Furan, 2-hydroxymethylfuran, and 2-acetylfuran are hepatotoxic. Furosemide, furan, 2,3-benzofuran and certain other furans produce acute renal tubular necrosis. Other simple furans such as 2-methylfuran, 3-methylfuran, and furan itself produce lung damage, and pulmonary edema (58). Thus, a variety of tissue lesions seen after the use of furan-containing compounds probably results from metabolic activation similar to that proposed for 4-ipomeanol and furosemide.

Cephaloridine

Extension of the furan studies to thiophene, another heteroaromatic derivative, has shown that several thiophene-containing compounds produce hepatic and renal necrosis (39). Cephaloridine (VI), a widely prescribed cephalosporin antibiotic, has its use limited primarily because of renal necrosis associated with such therapy (49). Pretreatments of mice with piperonyl butoxide and cobalt chloride decrease renal necrosis caused by cephaloridine. Further studies are in progress to determine if this specific renal necrosis is mediated by a reactive metabolite.

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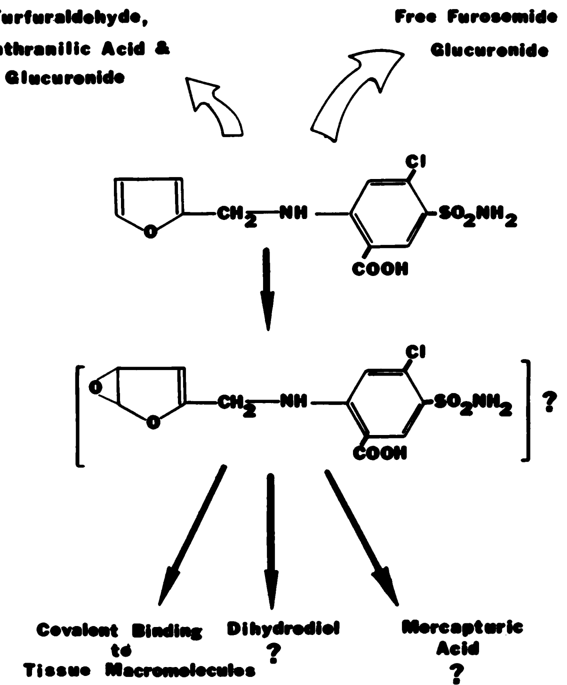

Figure 9. Possible reaction pathways for the metabolism of furosemide (See *Ref.* 52)

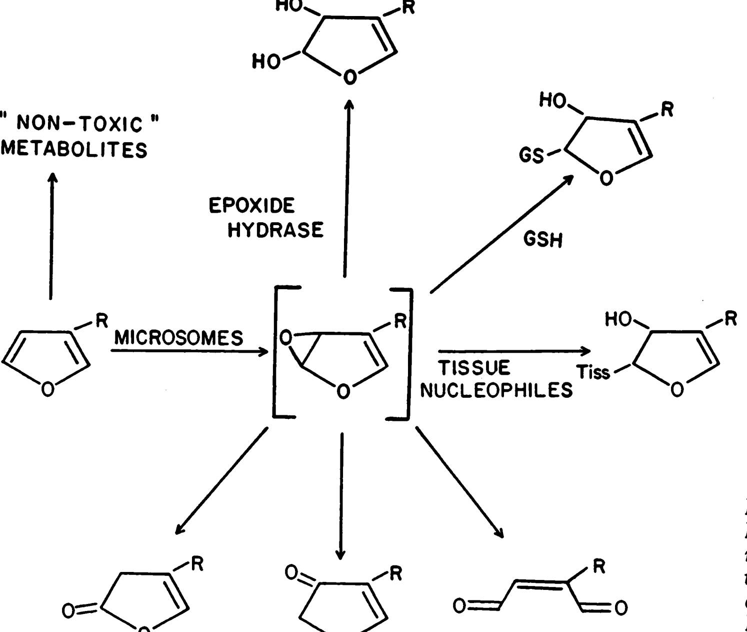

Figure 10. Possible mechanisms for the metabolism of furans (See *Ref.* 54)

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Conclusion

The general concepts discussed in this chapter which underlie the role of metabolic activation in chemical-induced tissue injury are the following: 1) First is the concept of reactive metabolite formation and whether the metabolite is important in manifesting a toxic reaction. 2) Second is the concept of target organ. Given that there is toxicity to a certain organ, is this due to metabolites formed in that tissue or elsewhere. Embodied in this concept is the important phenomenon, target organ switching. 3) Finally is the concept of dose-threshold for a toxic drug reaction. Some compounds such as acetaminophen, phenacetin and furosemide, as well as certain other furans and thiophenes, are relatively innocuous until a threshold dose is reached. Other compounds such as acetylisoniazid show evidence for tissue injury at almost all levels of dosage.

The parameters that we utilize to study such phenomena are tissue necrosis in the target organ, the irreversible binding of drug metabolites to the target organ - something we call covalent binding, the trapping of reactive metabolites and analysis of the pattern of drug metabolites.

To provide a biochemical basis for the toxic reactions, we alter the amounts and/or activities of enzymes responsible for the formation of reactive metabolites and look for correlations in the four parameters. It should be emphasized that with both inducers and inhibitors of metabolism it is difficult to predict _a priori_ their actual effect on a given toxicity _in vivo_, since they may affect both toxifying as well as detoxifying pathways. The situation is especially complex when more than one tissue can serve as a site for both toxification and detoxification, and when each tissue may respond differently to a given inducer or inhibitor. Whatever effect a particular pretreatment produces, however, the correlation between the severity of the toxicity and the degree of covalent binding of the toxin to the target tissue should be maintained if the two phenomena are related. Thus, manifestation of chemical-induced tissue injury depends on several critical kinetic parameters - namely, rate of formation, the inherent reactivity, the rate of detoxification (e.g. by glutathione conjugation) of toxic metabolites, and the rate of tissue repair by the injured organ.

While this work has as its goal the development of an integrated approach for determining biochemical mechanisms involved in the pathogenesis of chemical-induced tissue injury, several questions remain unresolved. The phenomenon of metabolic activation of chemicals to reactive intermediates which covalently bind to tissue macromolecules is well established as an initial step in leading to cell damage. But there is no explanation for why a particular reaction such as N-oxidation leads to tissue necrosis after the administration of one compound and neoplasia following another.

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Until the chemical-tissue interactions critical to particular necrotic or neoplastic processes are defined, a full assessment of meaning of the covalent binding of metabolites to tissue macromolecules cannot be made. For the present, it seems necessary to accept the fact that we cannot have new advances in drug therapy, for example, without some risk of causing structural tissue lesions, and to concentrate on assessing benefit/risk ratios.

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