Cytochrome P450 monoxygenases catalyze diverse oxidations, such as hydroxylation of aliphatic and aromatic carbons, epoxidation of olefins, N-dealkylation of amines, and O-dealkylation of ethers by activation of molecular oxygen (1). These enzymes play major roles in the metabolism of a wide variety of drugs and organic compounds (2–5). In addition, endogenous substances—such as fatty acids, steroids, fat-soluble vitamins, and prostaglandins—are transformed by the actions of cytochrome P450 (6).

Many metalloporphyrins have been synthesized in attempts to develop practical catalysts for oxidative reactions and used to elucidate the molecular mechanisms of biological oxygen atom activation and oxidation of substrates (7). We have used various metalloporphyrins as cytochrome P450 chemical models for studying drug metabolism (8–17).

Metabolism studies are crucial to evaluate the safety and effects of drugs, food additives, pesticides, and other industrial chemicals. Metabolites are usually obtained from biological samples—such as urine, bile, and liver microsomal reaction mixtures—but the amounts are small, and new metabolites and metabolic pathways can be difficult to identify. In addition, the toxicity of many chemicals is due to the initial formation and subsequent reactions of highly reactive metabolites that cause tissue injury (18, 19). However, it is difficult to detect such reactive metabolites directly in biological samples and to determine the origin of the toxicity, because most of them form adducts with cellular constituents, leading to cellular damage. For these reasons, we have applied a variety of cytochrome P450 models to study cytochrome P450 metabolism (8–17). Cytochrome P450 models are able to provide relatively large amounts of metabolites and reactive metabolites, and are also useful to analyze the metabolic mechanisms.

The present study was focused on the cytochrome P450-catalyzed conversion of p-substituted phenols. The metabolism of phenol derivatives is interesting, because most aromatic compounds are hydroxylated to phenol derivatives by cytochrome P450. We have already reported that p-phenoxyphe nol and p-methoxyphenol suffer “cleavage of the oxygen-aromatic ring bond” in cytochrome P450 models (8–17). It was also proved that this cleavage reaction is accompanied by ipso-substitution by the oxygen atom of the active species, and a hydroxy group of the substrate is necessary for this pathway to operate. On the basis of these results, we supposed that similar reactions might occur generally in various p-substituted phenols other than p-hydroxyaryl ethers (fig. 1). Various substrates might be replaced by the cytochrome P450 active species. Catechol derivatives formation and conjugation by UDP-glucuronosyltransferase or aryl sulfotransferase are well-known metabolic pathways of p-substituted phenols. ipso-Substitution might be the third metabolic pathway of p-substituted phenols.

We have recently shown that such a reaction proceeds in a cytochrome P450 model system that consists of meso-tetraakis(2,6-difluoro-phenyl)porphinoatoiron (III) chloride and mCPBA (16). These results encouraged us to examine whether this kind of oxidative reaction occurs with cytochrome P450 itself. Dehalogenation of halogenated phenols is known to be a cytochrome P450-mediated metabolic process (20–24) that also supports our hypothesis. Elimination of substituents other than halogen groups is a completely new type of metabolic pathway. It would be especially interesting if this type of reaction occurs with substrates whose substituents are attached to the aromatic ring through a carbon—carbon bond, such as p-acetylphenol, p-hydroxybenzyl alcohol, p-hydroxybenzoic acid, etc., because...
the carbon—carbon bond is very stable and has generally been thought to resist metabolism. We also report the results of the elimination of halogen groups for comparison.

We conducted this study to characterize the above metabolic pathway in rat liver microsomes and to study its mechanism in detail.

Materials and Methods

Chemicals. p-Toluquinol was prepared by the photooxygenation of p-cresol in the presence of Rose Bengal according to the method of Endo et al. (25). Similarly, 1-hydroxy-cis-7-oxabicyclo[4.3.0]nona-2-en-4-one was synthesized by the photooxygenation of 2-(4-hydroxyphenyl)ethyl alcohol (p-hydroxyphenethyl alcohol) (in the presence of Rose Bengal (25)), p-Hydroxyphenyl benzoate was synthesized by reaction of hydroquinone and benzoyl chloride in the presence of sodium carbonate (26). Phenyl p-hydroxybenzoate was obtained by treatment of sodium carbonate after reaction of p-hydroxybenzoic acid, phenol, and phosphorus oxychloride (27).

These four compounds were purified by silica gel column chromatography and identified on the basis of 1H-NMR and mass spectra.

NADP⁺ and G-6-P were purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and were stored at 4°C. G-6-P DHase (E.C. 1.1.1.49) from Baker’s yeast was purchased from Sigma Chemical Co. (St. Louis, MO) and was stored at −20°C. ¹⁸O₂ gas was from ISOTEC (Miamisburg, OH). All other chemicals were of the purest grade commercially available.

Preparation of Microsomes. Male Wistar rats (6 weeks old, 150–200 g each) were treated by intraperitoneal injection of phenobarbital (60 mg/kg in saline) for 3 days and killed 24 hr after the last injection. Hepatic microsomes were prepared as previously described (8). Microsomal protein concentration was determined by the Lowry et al. (28) method using bovine serum albumin as a standard, and the cytochrome P450 content was measured as described by Omura and Sato (29).

Microsomal Incubations. Detection of Hydroquinone and Quinols. The incubation mixture containing liver microsomes (4 mg protein), substrate (1 mM), KCl (60 mM), MgCl₂ (4 mM), G-6-P (4 mM), and G-6-P DHase (5 units) in 2.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) was preincubated for 3 min at 37°C. The reaction was initiated by adding NADP⁺ (final 0.4 mM). After incubation for 20 min at 37°C, the mixture was treated with 2 ml of ice-cold ethyl acetate to stop the reaction and extract the products, and the organic phase was separated and concentrated by argon flushing. Products formed were trimethylsilylated with BSTFA and pyridine.

Detection of Formylaldehyde (Substrate = p-Hydroxybenzyl Alcohol). This incubation was conducted under the same conditions as described. After incubation for 20 min at 37°C, the mixture was treated with 1 ml of 20% trichloroacetic acid aqueous solution to stop the reaction. Amounts of formylaldehyde formed were determined by Nash’s method (30, 31).

Detection of Benzoic Acid, Etc. (Substrate = p-Benzoylphenol). This incubation was conducted under the same conditions as described. After incubation for 20 min at 37°C, the mixture was treated with 1 ml of 2 N HCl aqueous solution to stop the reaction. Products were extracted with 2 ml of ethyl acetate, and the organic phase was separated and concentrated by argon flushing. Products formed were trimethylsilylated with BSTFA and pyridine.

Inhibition of Hydroquinone Formation by CO and Metyrapone. Inhibition by CO. This incubation was performed under the same conditions as described, except that it was done under an atmosphere of 20% O₂/80% CO.

Inhibition by Metyrapone. This incubation was performed under the same conditions as described, except it contained metyrapone (2 mM).

Microsomal Incubation Under ¹⁸O₂. This incubation was conducted under the same conditions as used for the microsomes-NADPH/O₂ system, except that it was done under an atmosphere of ¹⁸O₂/argon. The components, except NADP⁺, were contained in a two-necked flask, and NADP⁺ was separately placed in a glass vessel connected to the flask. The system was also connected by a glass tube to a vial containing 100 ml of ¹⁸O₂ gas (89 atom%), a balloon-containing argon gas, and a vacuum pump. Although immersed in an ice bath, the incubation mixture was evacuated 10 times, and the atmosphere was replaced with argon gas each time. After a final evacuation, the seal to the ¹⁸O₂ gas reservoir was broken, and the gas was allowed to distribute through the system, followed by the introduction of argon gas to equalize the pressure. The reaction was subsequently initiated by addition and mixing of the NADP⁺.

The ¹⁸O content in hydroquinone was calculated from the 256/254 (M⁺ + 2/M⁺) peak ratio in the mass spectrum of the trimethylsilylated derivative, then corrected to take account of the original ¹⁸O content of ¹⁸O₂ (14).

Results and Discussion

Oxidation of p-Substituted Phenols by Liver Microsomal Cytochrome P450. Based on the previous results of oxidation by the cytochrome P450 model (fig. 2A), various p- substituted phenols (X = F, Cl, Br, NO₂, CN, CH₃, CH₂OH, COCH₃, COPh, and COOH) were incubated with rat liver microsomes (NADPH/O₂ system). Figure 2B shows the results on substituent elimination from various p-substituted phenols to afford hydroquinone, which was identified as the trimethylsilylated derivative by GC/MS. However, the primary product, in some cases, may be p-benzoxquinone, not hydroquinone, because p-benzquinone is easily transformed to hydroquinone by reduction dependent on rat liver microsomes-NADPH (14).

All of the substituents, except X = CH₃, were eliminated, and relatively large amounts of hydroquinone were formed when p-halogenophenols (especially X = F), p-nitrophenol, p-hydroxybenzyl alcohol, and p-benzylphenol were used as substrates. Results obtained herein were similar to the results in the cytochrome P450 model system (fig. 2, A and B), implying that the cytochrome P450 model is a good mimic of cytochrome P450. It is interesting that this type of reaction occurred even in the case of some substrates whose substituents are attached to the aromatic ring through a carbon—carbon bond, (such as p-acetylphenol, p-hydroxybenzyl alcohol, p-hydroxybenzoic...
a different structure, or other cellular macromolecules. To apply this metabolic reaction to toluquinol is a toxic metabolite that binds covalently to DNA, RNA, step. This finding is significant for metabolism studies, because the group is difficult to eliminate, and the reaction stopped before this event.

Continued...

Values are expressed as means ± SE (N = 3).

When microsomes or NADP⁺ were omitted from the complete system, hydroquinone was not detected.

CO inhibition of hydroquinone formation was measured. Data shown in table 1 demonstrate that the formation of hydroquinone was significantly inhibited in the presence of 20% O₂/80% CO. Further, a cytochrome P450-specific inhibitor, metyrapone, also suppressed the formation of hydroquinone (table 1). These results indicate that the hydroxy group of cholesterol in steroidogenic tissues (i.e., the adrenal cortex, testis, and ovary) (33). However, the cleavage reaction in the present study is entirely different from such cases.

In the case of p-cresol, p-toluquinol was formed (3.56 ± 0.08 nmol/nmol P450) instead of hydroquinone (fig. 3). The product was identified as the trimethylsilylated derivative by GC/MS on the basis of its retention time and mass fragment pattern, compared with those of the synthesized authentic compound. This indicates that the methyl group is difficult to eliminate, and the oxygen atom of the active species on cytochrome P450. The substituent elimination occurred, accompanied with the replacement of the substituent by the oxygen atom of the active species, as in the case of the cytochrome P450 model (16). This type of reaction is often called “ipso-substitution.” When p-cresol was used as a substrate under ¹⁸O₂/argon mixtures, the ¹⁸O content in p-toluquinol formed was almost 100%. This indicates that the oxygen atom of the active species attacked the root of the methyl group, and the methyl group was not eliminated but remained.

Requirement of Hydroxy Group on Substrate. p-Substituted phenols, which have a phenolic hydroxy group at the p-position to the substituent, were used as substrates (as described herein), because we assumed that a hydroxy group at the p-position to the eliminated substituent might be required for the ipso-substitution, by analogy with the result obtained in the cytochrome P450 model (16). To

**TABLE 1**

<table>
<thead>
<tr>
<th>Substituent (X)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₂</td>
<td>49</td>
</tr>
<tr>
<td>CN</td>
<td>46</td>
</tr>
<tr>
<td>CH₃OH</td>
<td>39</td>
</tr>
<tr>
<td>COCH₃</td>
<td>56</td>
</tr>
<tr>
<td>COPh</td>
<td>62</td>
</tr>
<tr>
<td>COOH</td>
<td>42</td>
</tr>
</tbody>
</table>

Reaction mixtures contained 4 mg protein of microsomes, 1 mM substrate, 60 mM KCl, 4 mM MgCl₂, 4 mM G-6-P, 0.4 mM NADP⁺, and 5 units of G-6-P DHase in 0.1 M phosphate buffer (pH 7.4). The mixtures were incubated at 37°C for 20 min. Inhibition percentage was calculated by determining to what extent the yield in the complete system was depressed by the inhibitor.

a These samples were incubated under 20% O₂/80% CO.

b These samples contained metyrapone (2 mM).
The $^{18}$O content calculated from the 256/254 peak ratio in the mass spectrum of the trimethylsilylated hydroquinone and then corrected on the basis of the hydroxy group, namely microsomal reactions of confirm the requirement of a hydroxy group, we investigated the result obtained in the cytochrome P450 model system (16). These results show that a hydroxy group at the $p$-substituent toluene was used as the substrate (data not shown). These results show that a hydroxy group at the $p$-position is indispensable for elimination. This finding is the same as the result obtained in the cytochrome P450 model system (16).

**Studies on the Mechanism of ipso-Substitution.** On the basis of the data presented herein, we suggest in fig. 5 a possible mechanism for metabolic ipso-substitution. First, $p$-substituted phenols are hydroxylated at ipso-position with the same mechanism that we proposed previously (14, 16). This intermediate (i.e. quinol) breaks down to afford $p$-benzoquinone or hydroquinone, resulting in elimination of the substituent. We have a good basis for thinking that ipso-substitution proceeds via a quinol intermediate, in that we detected $p$-toluquinol instead of hydroquinone when $p$-cresol was used as a substrate. This finding indicates that ipso-substitution probably occurs through a quinol in the case of other $p$-substituted phenols.

There are two types of substituent elimination from the quinol intermediate. When the substituent is eliminated with acceptance of the C—$X$ bond electron pair, $p$-benzoquinone is formed and the eliminated group is an anion. We will hereafter use the term "type I elimination" to refer to this kind of reaction (fig. 5). When the substituent is eliminated with donation of the C—$X$ bond electron pair to the aromatic ring, hydroquinone is formed and the eliminated group is a cation. Hereafter, we will use the term "type II elimination" to refer to this kind of reaction (fig. 5).

It is a very interesting problem to determine which type of elimination (type I or type II) occurs with each ipso-substitution. This might depend on the nature of the substrate. We have already reported that the substituent is, in the case of $p$-alkoxyphenol and $p$-phenoxyphenol, eliminated as an alcohol form or a phenol form during ipso-substitution (14). Therefore, it was concluded that the ipso-substitution of these phenols proceeds via type I elimination. To elucidate the type of elimination for the other substrates, we further investigated the leaving groups for some $p$-substituted phenols that yielded a relatively large amount of hydroquinone ($p$-hydroxybenzyl alcohol, $p$-benzoylphenol, and $p$-nitrophenol).

**p-Hydroxybenzyl Alcohols.** To quantify HCHO, the Nash assay (a colorimetric determination for HCHO) was used when $p$-hydroxybenzyl alcohol was used as a substrate. Results are shown in table 3. The yield roughly corresponds to the amount of hydroquinone formation from $p$-hydroxybenzyl alcohol. This result demonstrates that the leaving group is HCHO. The substituent is eliminated with donation of the C—$CH_2$OH bond electron pair to the aromatic ring in this case (fig. 6).

Accordingly, we conclude that the ipso-substitution of $p$-hydroxybenzyl alcohol proceeds via type II elimination.

**$p$-Benzoylphenols.** Detection of PhCHO and PhCOOH was attempted by GC/MS, when $p$-benzoylphenol was used as a substrate. PhCHO was not observed at all, whereas PhCOOH was formed (table 3) in a yield roughly corresponding to the amount of hydroquinone formation from $p$-benzoquinol. In this case, the substituent is eliminated with donation of the C—$CO$Ph bond electron pair to the aromatic ring and, at the same time, is transformed to PhCOOH owing to the aqueous condition, in which $H_2O$ attacks the carbonyl group of the substituent (fig. 7). Accordingly, it may be concluded that the ipso-substitution of $p$-benzoylphenol proceeds via type II elimination.

Another possible mechanism can be considered for this elimination reaction. $p$-Benzoylphenol may be converted to the ester form ($p$-hydroxyphenyl benzoate), accompanied with Baeyer-Villiger rearrangement catalyzed by cytochrome P450, followed by enzymecatalyzed hydrolytic cleavage to give hydroquinone and PhCOOH.

The ester was not detected by GC/MS in the reaction mixtures, because it is unstable and hydrolyzed easily in this system.

If the ipso-substitution of $p$-benzoylphenol proceeds via Baeyer-Villiger rearrangement, another possible ester form (phenyl $p$-hydroxybenzoate) should also be formed. However, no products of hydrolysis of this ester, namely phenol and $p$-hydroxybenzoic acid, were formed. In addition, Baeyer-Villiger rearrangement does not require a hydroxy group at the $p$-position; but, the cleavage reaction did not occur when $p$-benzoyltoluene was used as the substrate. We conclude that the mechanism of the ipso-substitution of $p$-benzoylphenol is not via Baeyer-Villiger rearrangement but via the direct replacement of the substituent with the oxygen of the active species.

**$p$-Nitrophenols.** Detection of NO$_3$ was attempted, when $p$-nitro-
phenol was used as the substrate. To convert NO\textsubscript{3}\textsuperscript{-} into NO\textsubscript{2}\textsuperscript{-}, the reaction mixtures were reduced by cadmium after the incubation, and Griess assay was used. As a result, NO\textsubscript{2}\textsuperscript{-} was detected (as shown in table 3). When the Griess assay was used without reduction, no NO\textsubscript{2}\textsuperscript{-} was detected, indicating that the nitro group is eliminated as NO\textsubscript{3}\textsuperscript{-} via the type I mechanism, and NO\textsubscript{2}\textsuperscript{-} is eliminated from the aromatic ring and is transformed to NO\textsubscript{3}\textsuperscript{-} from the type II process (fig. 8A).

Two possibilities may be considered to explain the change in the extent of ipso-substitution with change in the halogen substituent. The first possibility is based on the consideration that the order of yields in p-halogenophenols (F > Cl > Br) inversely reflects the order of size of the halogen substituents (F < Cl < Br). The cytochrome P450 active species is more hindered in its approach to the ipso-position by a larger substituent such as bromine. The second possibility was suggested by Cnubben et al. that the change in dehalogenation with change in the halogen substituent is due to an intrinsic electronic parameter of various p-halogenophenols dependent on the halogen substituent. The order of yields in p-halogenophenols (F > Cl > Br) corresponds to the order of electronegativity of halogen substituents (F > Cl > Br).

It is not clear what causes the observed change in dehalogenation, but these findings are extremely important for the following reason. A C—F bond is generally thought to be inert and difficult to break, and incorporation of a fluorine substituent into drugs has been used as a means of blocking biodegradation and bioactivation of the compounds. However, our findings show that a fluorine substituent attached to an aromatic ring can be metabolically eliminated to substantial extent.

In conclusion, we have shown that the substituent elimination of...
various p-substituted phenols, which is a novel metabolic pathway catalyzed by cytochrome P450, occurs in a rat liver microsomal system in accordance with results obtained in a cytochrome P450 chemical model system. Thus, the use of cytochrome P450 models as an approach to metabolism research seems to be effective for discovering novel metabolic pathways and analyzing their metabolic mechanisms.

It is interesting to note that this reaction occurs even in the case of some substrates whose substituents are attached to the aromatic ring through a carbon—carbon bond, because the carbon—carbon bond had been thought to be very stable and resistant to metabolism. It was proved that the substituent elimination is accompanied with ipso-substitution by the oxygen atom of the active species, and a hydroxy group at the p-position to the substituent to be eliminated is necessary for this pathway. The elimination can be divided into two types: type I (anion) or type II (cation) elimination.

Finally, there are two points that we should emphasize. The first is that hydroquinone or p-benzoquinone, a highly toxic metabolite, is formed in this metabolic pathway. Therefore, these findings may have important implications for drug metabolism studies and could help to explain some of the side effects of certain drugs. The second is that this metabolic pathway is applicable to a wide range of drugs and environmental chemicals, because most aromatic compounds are hydroxylated to phenol derivatives by cytochrome P450. Further studies of the occurrence of this metabolic pathway with a variety of compounds are in progress.

### Table 4

<table>
<thead>
<tr>
<th>Substituent X</th>
<th>ipso-Substitution</th>
<th>Hydroxylation</th>
<th>Total Amount</th>
<th>nmol/mmol P450</th>
<th>nmol/mmol P450</th>
<th>nmol/mmol P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>5.08 ± 0.61</td>
<td>8.48 ± 0.24</td>
<td>13.6 ± 0.80</td>
<td>0.60 ± 0.02</td>
<td>15.2 ± 0.47</td>
<td>15.8 ± 0.49</td>
</tr>
<tr>
<td>Cl</td>
<td>0.49 ± 0.02</td>
<td>17.2 ± 0.90</td>
<td>17.7 ± 0.88</td>
<td>0.80</td>
<td>18.0 ± 0.90</td>
<td>18.8 ± 0.90</td>
</tr>
</tbody>
</table>

Reaction conditions were the same as those of fig. 2. Products were quantified by GC/MS as trimethylsilylated derivatives. Total amounts were calculated as the sum of the values obtained for ipso-substitution and o-hydroxylation. Values are expressed as means ± SE (N = 3).

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**References**