Determination of Partition Ratios for Allylisopropylacetamide during Suicidal Processing by a Phenobarbital-induced Cytochrome P-450 Isozyme from Rat Liver*

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Allylisopropylacetamide is shown to be a suicide substrate for the phenobarbital-inducible cytochromes P-450. In phenobarbital-induced rat liver microsomes about 70% of the cytochrome P-450-mediated N,N-dimethylaniline N-demethylase activity is sensitive to allylisopropylacetamide inactivation; the residual 30% of the N-demethylase activity is incapable of allylisopropylacetamide turnover and insensitive to allylisopropylacetamide inactivation. The partition number for inactivation of the susceptible population of cytochrome P-450 indicates turnover of 201 molecules of allylisopropylacetamide per molecule of P-450 inactivated. A purified phenobarbital-induced isozyme of cytochrome P-450, when reconstituted with purified rat liver cytochrome P-450 reductase, is also inactivated by allylisopropylacetamide in a suicide fashion with a corrected partition ratio of 184 turnovers of allylisopropylacetamide per inactivation event. This partition number is corrected for the competing O₂-dependent autoinactivation of cytochrome P-450 which we have previously shown to occur with the purified isozyme (Loosemore, M., Light, D. R., and Walsh, C. (1980) J. Biol. Chem. 255, 9017–9020). The 201 product molecules of cytochrome P-450-mediated turnover of allylisopropylacetamide in either the microsomal or purified enzyme system are probably the epoxide, are reactive toward alkylation of cellular nucleophiles, and covalently modify protein and exogenous calf thymus DNA molecules.

Allyl-substituted drugs are known to cause isozyme-specific destruction of cytochrome P-450 monoxygenase in the liver of phenobarbital-induced rats (1–9) and so generate green pigments in that organ. These are green heme derivatives which result from stoichiometric N-alkylation of one of the heme tetrapyrrole nitrogens during P-450 inactivation by such compounds as secobarbital, allylisopropylacetamide, and sedormid (10, 11). Elegant structural studies on the heme adducts by Ortiz de Montellano and Kunze (12) have confirmed that olefins and alkenes as simple as ethylene and acetylenes produce such adducts. Oxygen transfer to C–C multiple bonds by the phenobarbital-induced liver P-450 isozyme group can clearly lead to autoinactivation by mechanism-based routes.

In contrast to the recent structural advances, the kinetics of monoxygenase inactivation by such unsaturated substrate molecules either in microsomal vesicles or with pure heme protein have been relatively little studied. White recently reported P-450 destruction with first order half-times of 30 min and 9 min for acetylenes such as danazole and dipropargylacetamide (13). We recently reported the Kᵢ for the paradigmatic allylic olefin allylisopropylacetamide to be 80 μM in phenobarbital-induced rat liver microsomes and a kᵢ₅ₐ₅ of about 0.1 min⁻¹ (half-time = 7.3 min). These studies did not specifically determine that such inactivators are also processed as substrates, although there are data with allylisopropylacetamide, for example, in whole rats (14) and in perfused rat liver (15), showing metabolism to products which are acerbable to an initial epoxide product from P-450 oxygenative action.

In this report we have examined the substrate behavior of 2-[¹⁴C]allylisopropylacetamide with phenobarbital-induced rat liver microsomes and a purified phenobarbital-induced cytochrome P-450 isozyme and determined the partition ratio for allylisopropylacetamide as a suicide substrate, the number of times products are released per inactivation event (16–19) (Scheme 1). This is clearly a measure of efficiency as a mechanism-based isozyme-specific inactivator. We have examined the electrophilic reactivity of the presumed epoxide product by analysis of radioactivity incorporated into protein and into exogenously introduced calf thymus DNA.

Experimental Procedures

Materials—Calf thymus DNA, Sephadex G-10, bovine serum albumin, morpholinopropanesulfonic acid, 4-(2-hydroxyethyl)-1-ethanesulfonic acid, tris(hydroxymethyl)aminomethane, sodium cholate, di-lauroylphosphatidylcholine, deoxycholic acid, phenobarbital, and NADPH were purchased from Sigma. [³⁵S]dimethylamine was prepared as previously described (20). Allylisopropylacetamide and 2-[¹⁴C]allylisopropylacetamide were gifts from Hoffmann-La Roche Inc.

Methods—The [³⁵S]dimethylamine synthesis and assay were as previously described, as were all enzyme purifications (the phenobarbital P₄₅₀ isozyme and reductase) and reconstitutions of pure P₄₅₀ enzyme (20). All microsomal studies used phenobarbital-induced rat liver microsomes prepared as previously stated (20).

Allylisopropylacetamide metabolites were converted to their lactones as described by Doedens (14) by treatment of the aqueous reaction phase with mineral acid at pH < 2 on a steam bath for 1 h. Typically this gave a recovery of radioactivity upon extraction of the aqueous phase with ethyl acetate of better than 80%.

Aliquots of the concentrated ethyl acetate phase were then injected onto an 8% OV-17 column at 150 °C on a Perkin-Elmer model No. 390B gas chromatograph. Fractions were collected in liquid nitrogen chilled glass tubes and washed into scintillation vials for determination of ³⁵S radioactivity. Typical recoveries of radioactivity from the gas chromatograph were 75–80%.

Authentic samples of allylisopropylacetamide 4,5-diol and lactones.
Suicidal Processing of Allylisopropylacetamide by Rat P-450

**RESULTS**

*Extent of Allylisopropylacetamide-mediated Cytochrome P-450 Inactivation in Induced Rat Liver Microsomes*—To calibrate the kinetics and extent of cytochrome P-450 destruction by allylisopropylacetamide seen by previous workers as well as in our own work (10, 11, 20), phenobarbital-induced rat liver microsomes (1-5 nmoles of cytochrome P-450/ml) were incubated with 2.0 mM allylisopropylacetamide and 1-3 mM NADPH at 37°C with shaking and open to the air. Consistent with expectations (10, 11, 20), the resultant rapid loss of 63 ± 12% of the initial microsomal P-450 dimethylaniline N-demethylase activity occurred in a first order manner with a half-life of 7.3 ± 0.3 min (Fig. 1A). Approximately 30-40% of the microsomal cytochrome P-450 N-demethylase activity was found to be resistant to inactivation by allylisopropylacetamide over the next 2 h of incubation.

*Microsomal Metabolism of Allylisopropylacetamide by Cytochrome P-450 in Competition with Inactivation*—Two parallel incubations (4.0 ml each) of microsomal cytochrome P-450 (10 nmol/ml) with allylisopropylacetamide (2.0 mM) in the presence of 1-3 mM NADPH and open to the air were performed. One contained 2-[14C]allylisopropylacetamide (3.55 Ci/mol) and the other contained unlabeled allylisopropylacetamide. The reaction with unlabeled allylisopropylacetamide was followed by monitoring dimethylaniline N-demethylation activity, and when this procedure indicated a loss of 20% of the initial N-demethylase activity (corresponding to 8.1 nmol of cytochrome P-450 inactivated) the parallel 2-[14C]allylisopropylacetamide reaction was quenched by freezing. The [14C]allylisopropylacetamide-derived metabolites formed were converted to the 2-[14C]allylisopropylacetamide-lactones and quantitated by radio-gas chromatographic analysis as shown in Fig. 24. This figure shows the formation of both the 5-membered \( \gamma \)-lactone and the 6-membered \( \delta \)-lactone from the chemical cyclization procedure as peaks with retention times of 10.5 and 11.2 min. Unmetabolized allylisopropylacetamide gives a retention time of 7.5 min; the peak with retention time of 4.0 min was shown to arise nonenzymatically from treatment of allylisopropylacetamide by the same procedure used to convert metabolites to lactones (Fig. 2C). This unidentified peak was shown not to be allylisopropylacetamide (1), allylisopropylacetamide-4,5 diol (3), either allylisopropylacetamide-lactone (4, 5) or allylisopropylacetamide-4,5-diol acid (see Scheme 4 for corresponding structures) by comparison with the gas chromatographic retention times of synthetic standards, and in the product analysis was not treated as an enzymic metabolite of allylisopropylacetamide. Making the assumption that the allylisopropylacetamide-derived small molecule metabolites are all converted to the lactone mixture (14), we can calculate that the [14C]lactone products indicated a turnover of 1.63 \( \mu \)mol of allylisopropylacetamide in the radioactive incubation. Given an allylisopropylacetamide-induced destruction of 8.1 nmol of cytochrome P-450 in this interval, as noted above, we can calculate the partition ratio to be 201 product molecules formed for every molecule of cytochrome P-450 inactivated by allylisopropylacetamide molecule in turnover (Table 1).

**Scheme 1**

![Scheme 1](image)

**Allylisopropylacetamide**

- **E₁** → FAD, FMN
- **E₂** → HEME

**NADPH** + **NADP**

\( O_2 \) + **H₂O**

**PRODUCTS**

**INACTIVE**

**ENZYME**

Allylisopropylacetamide was repeatedly acid-washed were resuspended in distilled acetic acid. Centrifugation then pelleted the protein. Samples which were prepared by chemical oxidation of allylisopropylacetamide and postion by allylisopropylacetamide seen by previous workers as well as in our own work were removed by spooling on a glass rod. The DNA was then successively lactonization as described by Doedens (14) and gave NMR and IR spectra consistent with those reported for these compounds (14). Allylisopropylacetamide and \([14C]\)allylisopropylacetamide were used as obtained. These standards were cojuncted with enzyme reaction mixtures to aid in identification.

Protein precipitations were routinely performed by adding sufficient trichloroacetic acid to make the reaction mixture 1% in trichloroacetic acid. Centrifugation then pelleted the protein. Samples which were repeatedly acid-washed were resuspended in distilled H₂O, then made 1.0 M in HCl, vortexed, and centrifuged. Determinations of protein were as described by Lowry et al. (21).

DNA isolation was performed by making the enzyme reaction mixture 0.3 M in NaCl, 0.8% in sodium dodecyl sulfate, and then extracting with an equal volume of dichloromethane. Samples were then centrifuged and the aqueous layer pipetted off and chilled on ice. Three volumes of cold ethanol were then added and the DNA removed by spooling on a glass rod. The DNA was then successively washed with about 3 ml each of ethanol, benzene, dichloromethane (2 times), ethanol (2 times), diethyl ether (3 times), and dried overnight in a dessicator. The DNA was then dissolved in HC1, vortexed, and centrifuged. Determinations of protein precipitations were routinely performed by adding sufficient trichloroacetic acid to make the reaction mixture 10% in trichloroacetic acid. Centrifugation then pelleted the protein. Samples which were repeatedly acid-washed were resuspended in distilled H₂O, then made 1.0 M in HCl, vortexed, and centrifuged. Determinations of protein were as described by Lowry et al. (21).

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30% of the initial N-demethylase activity was reached and further incubation had been shown to have no effect on this residual N-demethylase activity. At this point, [14C]allylisopropylacetamide at 7.1 Ci/mol was added at the level of 2 μmol/ml of incubation to make the allylisopropylacetamide residual N-demethylase activity. At this point, further incubation had been shown to have no effect on this 30% of the initial N-demethylase activity was reached and virtually none of the capacity to metabolize allylisopropylacetamide persisted. The partition frequency of this P-450 population toward allylisopropylacetamide was not determined because of this very slow rate of utilization and lack of detectable inactivation.

Metabolism of Allylisopropylacetamide by Purified Isozyme from Phenobarbital-induced Rat Liver Microsomes in the Reconstituted System—The cytochrome P-450 isozyme, purified from phenobarbital-induced rat liver microsomes as an N,N-dimethylaniline demethylase (20), was reconstituted in a 1:1 ratio, with pure rat liver cytochrome P-450 reductase (20) at the level of 2.5 nmol of P-450 and reductase. The reconstituted system contained, along with the P-450 and reductase, 0.03% dilaurylphosphatidylcholine (sonicated), 0.01% sodium deoxycholate, and small amounts of cholate and Renex 690 (polyoxyethylene 10-nonyl phenyl ether), introduced into the mixture with the enzyme samples, in a volume of 2.0 ml with 0.1 M, pH 7.5, morpholinopropanesulfonic acid buffer. This system was incubated at 37 °C open to the air with shaking in the presence of 1.1 mM NADPH, 2.1 mM [14C]allylisopropylacetamide (3.55 Ci/mol) for 30 min. (The reconstituted system no longer catalyzed N-demethylation of dimethylamine at 30 min.) We have previously shown that under these conditions rat liver cytochrome P-450 is 100% inactivated even in the absence of allylisopropylacetamide due to autoinactivation by a reduced oxygen metabolite arising in uncoupled turnover from NADPH oxidation (20). In the presence of an oxygenatable substrate such as dimethylamine or allylisopropylacetamide, oxygen transfer to substrate is competitive with this autoinactivation (20).

The reaction was terminated at 30 min by addition of 100 μl of concentrated HCl and the protein was removed by centrifugation. Analysis of the aqueous supernatant for allylisopropylacetamide metabolites was carried out with the resulting radio gas chromatographic trace shown in Fig. 2B. The total [14C]allylisopropylacetamide-lactone produced is equivalent to 92 molecules of allylisopropylacetamide for each molecule of cytochrome P-450 inactivated (Table I).

1 The actual concentration and specific activity of allylisopropylacetamide were likely to have been different, with the extremes being 2 mM and 7.1 Ci/mol, if all of the unlabeled allylisopropylacetamide had been previously consumed, and 4.4 mM and 3.2 Ci/mol if 201 molecules of allylisopropylacetamide were consumed, per inactivated cytochrome P-450 with no metabolism by residual cytochrome P-450. The [14C]allylisopropylacetamide specific activity used for product determination was 3.5 Ci/mol, which would result in a 2-fold overestimation of product if the former case is true or a low estimate by 14% in the latter case.
Suicidal Processing of Allylisopropylacetamide by Rat P-450

Binding of [14C]Allylisopropylacetamide to Protein in Microsomal and Purified Cytochrome P-450 Isozyme Incubations—The partition ratio of products:cytochrome P-450 inactivation event produced from the microsomal or the pure phenobarbital-induced P-450 incubations is based on an assay for low molecular weight metabolites. Since the epoxide is the likely initial oxygenation product, one might expect that in competition with hydrolysis to the diol, it could serve as an alkylating agent for cellular macromolecules. Protein alkylation was analyzed first. Protein obtained from the incubation of microsomes with 2-[14C]allylisopropylacetamide, which was terminated at the point of inactivation of 30% of the N-demethylease activity (see Table II), was collected and analyzed for bound 14C radioactivity.

The protein was repeatedly acid-precipitated until less than 1% of the radioactivity found in the first wash was present in the supernatant. The sample of protein was then resuspended in 0.1 M morpholinopropanesulfonic acid, pH 7.5, buffer and centrifuged to remove suspended particles. The clear sample containing 65% of the acid-washed protein was then subjected to chromatography on Sephadex G-10 in pH 7.5, 0.05 M Tris-HCl buffer. A single coincident peak of radioactivity and absorbance at 280 nm eluted.

Protein determination of the peak fractions showed that there were 1.5 ± 0.2 nanomoles of [14C]allylisopropylacetamide-derived radioactivity/mg of protein (Table II).

Identical treatment of the protein obtained from the incubation of [14C]allylisopropylacetamide with the reconstituted system containing the purified cytochrome P-450 isozyme also gave a single peak on the Sephadex G-10, which after Lowry protein determination showed 0.67 ± 0.14 nmol of [14C]allylisopropylacetamide-derived radioactivity/mg of protein (21). This treatment of the protein registers only acid-stable protein labels. Since the modified heme group is acid-labile (22, 23) and is removed in the acid wash supernatant, these data reflect allylisopropylacetamide labeling of the apoprotein.

[14C]Allylisopropylacetamide Binding to DNA with Purified Phenobarbital-induced Cytochrome P-450 in the Reconstituted System—To determine whether any of the oxygenated product molecules could also modify DNA covalently, a standard protocol (24) was used with calf thymus DNA added as the model DNA. The purified phenobarbital-induced isozyme was reconstituted as described at 2.5 nmol of P-450, 0.8 mg of calf thymus DNA, and 4 µmol of [14C]allylisopropylacetamide (3.55 Ci/mol) in 2.0 ml of 0.1 M, pH 7.5, morpholinopropanesulfonic acid at 37°C. After 45 min, the reaction was stopped and DNA isolated as described under "Experimental Procedures." Analysis for bound 14C radioactivity showed 0.32 ± 0.3 neq of [14C]allylisopropylacetamide/mg of DNA. Using the value of ECM mol = 6600 for calf thymus DNA (25) for quantitation of the DNA samples, we find that this level of bound 14C radioactivity indicates 1.0 molecules of [14C]allylisopropylacetamide-derived radioactive species per 8.8 ± 0.7 x 10^6 nucleotides (Table II).

[14C]Allylisopropylacetamide Binding to DNA Microsomal Incubations—Microsomes incubated with [14C]allylisopropylacetamide and the synthetic lactones were coinjected as mass sources, and the bars represent recovered radioactivity. As noted in the text, the peak at 4' is an unknown, at 7.5' allylisopropylacetamide, at 10.5' the γ-lactone, and at 11.2' the δ-lactone. Overall recoveries were as described in the text. B, the reaction products from the incubation of [14C]allylisopropylacetamide with the purified phenobarbital-induced rat liver isozyme, obtained as described in the text. The reconstitution of the pure P-450 and incubation procedures were also as described in the text. The figure represents injection of 3 µl of a 4.0-ml ethyl acetate solution of the recovered reaction. Conditions are the same as A. C, authentic [14C]allylisopropylacetamide was treated by the lactonization process described in the text for analysis of metabolites. 3.0 µl of a 2.0-ml ethyl acetate solution were injected, and the data are displayed as in A.

**FIG. 2.** Products of allylisopropylacetamide metabolism. A, radio-gas chromatograph trace of [14C]allylisopropylacetamide metabolites formed upon incubation with microsomal cytochrome P-450. The reaction products from the incubation of [14C]allylisopropylacetamide with microsomal cytochrome P-450 were isolated as described in the text. 3.0 µl of a 3.0-ml ethyl acetate solution of the recovered reaction was analyzed for bound 14C radioactivity. The solid line is the gas chromatographic trace; authentic metabolites formed upon incubation with microsomal cytochrome P-450. As noted in the text, the peak at 4' is an unknown, at 7.5' allylisopropylacetamide, at 10.5' the γ-lactone, and at 11.2' the δ-lactone. Overall recoveries were as described in the text. B, the reaction products from the incubation of [14C]allylisopropylacetamide with the purified phenobarbital-induced rat liver isozyme, obtained as described in the text. The reconstitution of the pure P-450 and incubation procedures were also as described in the text. The figure represents injection of 3 µl of a 4.0-mi ethyl acetate solution of the recovered reaction. Conditions are the same as A. C, authentic [14C]allylisopropylacetamide was treated by the lactonization process described in the text for analysis of metabolites. 3.0 µl of a 2.0-ml ethyl acetate solution were injected, and the data are displayed as in A.
suicide Processing of Allylisopropylacetamide by Rat P-450

Sopropylacetamide and calf thymus DNA similarly resulted in $^{14}$C radioactive binding to DNA in a P-450 enzyme-dependent manner. Time dependence of radioactive labeling of exogenous DNA was tested by incubating microsomes (2.5 nmol of P-450/ml) with $[^{14}$C]allylisopropylacetamide (3.55 Ci/mol, 1.0 mm), 0.8 mg of DNA/ml, 15 mM MgCl$_2$, and 1-3 mM NADPH at 37°C in a total of 5.5 ml of 0.1 M, pH 7.5, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The reaction was started by the addition of the NADPH and 1.0-ml aliquots were removed with time and the DNA of each isolated and analyzed for radioactivity incorporated. As shown in Fig. 3A, the level of incorporation of $^{14}$C radioactive/mg of DNA increased linearly over the initial 10-20 min of incubation and then began to level off. It was found routinely under these conditions that a 45-min incubation would result in levels of 1.0 equivalent of $[^{14}$C]allylisopropylacetamide incorporated per 4500 nucleotides (Table II). A blank containing all of the above, with the exception that the microsomes were inactivated by boiling, was used to account for $[^{14}$C]allylisopropylacetamide label incorporation not due to metabolism (blank values were about 5% of the sample signal). Chromatography of the labeled DNA on Sephadex G-10 in 0.05 M Tris-HCl, pH 7.5, followed by limited acid hydrolysis with 2 N formic acid for 2 h, followed by a second Sephadex G-10 chromatography step results in a distinct DNA peak containing one $[^{14}$C]allylisopropylacetamide-derived equivalent/1200 nucleotides, a 3-4 fold enrichment (1/4500 (Table II) going to 1/1200) over the initial adduct level in DNA. This rise in adduct level per nucleotide is the pattern expected for covalently derivatized DNA and would not be expected if the associated label had been noncovalently associated.

Radioactive Metabolite Binding to DNA Is Abolished by Preincubation of P-450 with Unlabeled Allylisopropylacetamide—To validate that the radioactive metabolite of $[^{14}$C] allylisopropylacetamide reactive for DNA alkylation was produced by the P-450 monooxygenase, we demonstrated that preincubation of microsomes with unlabeled allylisopropylacetamide for various times before the $[^{14}$C]allylisopropylacetamide was introduced reduced subsequent labeling of DNA with a kinetic pattern similar to that of P-450 inactivation (Fig. 1, A and B). Microsomal cytochrome P-450 (5 nmol/ml) was incubated with allylisopropylacetamide (1.0 mm) in 0.1 M morpholinopropanesulfonic acid, pH 7.5, buffer (final incubation volume, 3.0 ml) and 1-3 mM NADPH at 37°C. At various times 0.5-ml aliquots were removed and diluted 1:1 into 0.1 M, pH 7.5, morpholinopropanesulfonic acid buffer containing 1.0 mm $[^{14}$C]allylisopropylacetamide (3.55 Ci/mol) and 0.8 mg of DNA. The diluted samples were then incubated again at 37°C with 1.0 mM NADPH for 45 min. The DNA was then isolated and analyzed for $[^{14}$C]allylisopropylacetamide incorporation. The data shown in Fig. 1B indicate that

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**TABLE II**

**Binding of $[^{14}$C]allylisopropylacetamide-derived metabolites to protein and to DNA**

The protein and DNA recovered from incubations of $[^{14}$C]allylisopropylacetamide with microsomal cytochrome P-450 or the purified P-450 isozyme in the reconstituted system were isolated and analyzed for bound $[^{14}$C]allylisopropylacetamide-derived radioactivity as described in the text. The data shown represent bound $[^{14}$C]radioactivity as nanomole equivalents of $[^{14}$C]allylisopropylacetamide-derived radioactivity per mg of protein or as the number of DNA nucleotides per equivalent of $[^{14}$C]radioactivity derived from $[^{14}$C]allylisopropylacetamide based upon quantitation of protein or DNA as described in the text and the specific radioactivity of the $[^{14}$C]allylisopropylacetamide used in the incubations.

<table>
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<th>Form of cytochrome P-450</th>
<th>DNA nucleotides</th>
<th>$[^{14}$C]allylisopropylacetamide</th>
<th>DNA nucleotides</th>
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</thead>
<tbody>
<tr>
<td>Microsomal (phenobarbital-induced)</td>
<td>1.5 ± 0.2</td>
<td>4000-5000:1</td>
<td></td>
</tr>
<tr>
<td>Purified cytochrome P-450 isozyme from phenobarbital-induced rat liver microsomes</td>
<td>0.67 ± 0.14</td>
<td>8000-9000:1</td>
<td></td>
</tr>
</tbody>
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**Fig. 3.** Radioactive metabolite binding to DNA. A, increase in the level of binding of $[^{14}$C]allylisopropylacetamide metabolites to DNA as a function of time of incubation. Microsomal cytochrome P-450, $[^{14}$C]allylisopropylacetamide, and DNA were incubated as described in the text. At the times shown aliquots were removed and the level of $[^{14}$C]allylisopropylacetamide metabolites per mg of DNA determined as described under "Methods." The level of $[^{14}$C]allylisopropylacetamide-derived radioactivity per mg of DNA is plotted versus the time at which the DNA aliquot was removed from the incubation. B, SKF-525-A inhibition of microsomal cytochrome P-450 catalyzed binding of $[^{14}$C]allylisopropylacetamide to DNA. Incubations of $[^{14}$C]allylisopropylacetamide plus DNA were performed with and without SKF-525-A under the conditions described in the text. Aliquots were removed at various times and the DNA was isolated and analyzed as described for A. The incubation without SKF-525-A present is shown by C, and that with SKF-525-A is represented by Δ.
the preincubation with unlabeled allylisopropylacetamide leads to a rapid inactivation of subsequent $[^14]C$ allylisopropylacetamide incorporation into DNA with a half-time of approximately 9 min.

SKF-525-A Inhibition of Microsomal P-450 Catalyzed Incorporation of $[^14]C$ Allylisopropylacetamide into DNA—As a final index that the observed DNA labeling was due to P-450 monoxygenase activation and processing of allylisopropylacetamide, the classical P-450 enzyme inhibitor, SKF-525-A (26), was employed. DNA, 0.8 mg/ml, $[^14]C$ allylisopropylacetamide (3.55 Ci/mol), 2.0 mm, 2.0 mM NADPH, and microsomal cytochrome P-450 (20 nmol/ml) were incubated in 0.1 M, pH 7.5, morpholinopropanesulfonic acid (final volume, 4.5 ml) in the presence and absence of 3.5 mM SKF-525-A (shown to be 3 times higher than the minimum concentration required to inhibit completely the microsomal cytochrome P-450 N-demethylation of $[^14]C$ N,N-dimethylaniline and benzphetamine known to be specific for the phenobarbital-induced isozyme. This partial but not complete loss of microsomal N-dealkylation capacity is typical for a substrate supposedly inactivating olefins (or acetylenes) never cause quantitative inactivation even when assayed against a substrate supposed to be oxidatively demethylated of such substrates as N,N-dimethylaniline and benzphetamine known to be specific for the phenobarbital-induced isozyme. This partial but not complete loss of microsomal N-dealkylation capacity is typical for a whole series of olefinic and acetylenic inactivators (11, 12, 13).

Those P-450 enzyme molecules inactivated show modified heme spectra due to conversion of the heme cofactor to a green pigment which has the stoichiometry of a 1:1 adduct of heme cofactor, the olefinic or acetylenic substrate, and one oxygen atom from molecular oxygen (12). Impressive structural detective work has indicated that one of the tetrapyrrole nitrogen of the heme alkylatively intercepts a reactive oxygenated intermediate in a suicidal event from the cytochrome P-450 active site (11, 12).

Left unaddressed has been the question of how often this suicidal interception occurs compared to completion of the oxidative catalytic cycle and release of product in any of these olefinic or acetylenic inactivator cases. On the other hand, Smith (15) has reported a study on metabolism of $[^14]C$ allylisopropylacetamide in intact male rats as well as in perfused rat livers under conditions of phenobarbital induction. Extensive metabolic enureses with two major metabolites identified as the glycol (the 4,5 diol) of allylisopropylacetamide, presumably arising from epoxidation and then hydrative opening, and the y-lactone derived from the glycol. A time course of metabolism allowed Smith to recover, by 48–96 h, 68% of the $[^14]C$ radioactivity in the urine and 11% in the feces, via enterohelical circulation (15). The remaining radioactivity was in the liver (20%, up to 3 nmol of $[^14]C$/mg of liver) and in the kidney (6%). At early incubation times in perfused rat liver, there was a biphasic loss of heme P-450, with $t_{1/2}$ for the rapid component of 4.4 min and 3.3 h for a slower component, consonant with earlier observations (3, 27).

With this perspective one expects that in kinetic competition with the hepatotoxic suicidal handling of allylisopropylacetamide by susceptible P-450 isozymes, epoxide product molecules are produced and released. In this paper we have now quantitated this partition frequency by analyzing the $[^14]C$ product molecules directly while simultaneously monitoring the suicidal inactivation. In phenobarbital-induced microsomes the partition ratio is about 200:1, while it is 92:1 with a pure P-450 isozyme active for N-demethylation in a reconstituted system (Table I). The 92:1 ratio is undoubtedly low because we have recently shown that this isozyme is partly uncoupled in turnover, only 30–40% of the NADPH molecules oxidized are spent for substrate oxygenation. The rest of the flux results in $Q_{O_2}$ to $H_2O_2$ reduction, the uncoupling leading to enzyme autoinactivation with a $t_{1/2}$ of 7 min (20). Since we see a similar $t_{1/2}$ of 7–9 min for inactivation of dimethylaniline N-demethylation activity in microsomes specifically by allylisopropylacetamide, it is likely that in the reconstituted pure P-450 isozyme system, the P-450 molecule dies with about equal frequency from allylisopropylacetamide-induced heme cofactor alkylolation or from autoinactivation by a reduced oxygen metabolite in uncoupled flux. Thus $92 \times 2 = 184$ turnovers/inactivation might be a reasonable corrected partition ratio for those inactivations specifically mediated by allylisopropylacetamide with this pure P-450 isozyme. Such a value of 184 compares reasonably with the 201 partition frequency seen in microsomes.

In a simple scheme for suicidal processing, the turnover/inactivation ratio is equivalent to the ratio of $k_{cat}/[S]$ (17, 19). Since $k_{cat} = 0.1$ min$^{-1}$ in microsomes, $k_{cat} \approx 20$ min$^{-1}$.

**Scheme 2**

The extrapolation in the pure enzyme reconstitution experiments would be 18 min$^{-1}$.

The question arises why allylisopropylacetamide and other inactivating olefins (or acetylenes) never cause quantitative inactivation even when assayed against a substrate supposedly characteristic for a phenobarbital isozyme. The two limiting possibilities are (a) all susceptible P-450 molecules are alkylated at the heme group, but still retain 30% of uninhibited $V_{max}$ activity when assayed for dimethylaniline or benzphetamine N-demethylation; or (b) there are two or more P-450 species catalyzing N-demethylation, but only one recognizes allylisopropylacetamide as a substrate and only that one self-destructs during allylisopropylacetamide turnover. Our results favor the latter interpretation, since $[^14]C$ allylisopropylacetamide processing capacity is essentially eliminated while dimethylaniline is still oxygenatively demethylated.

In this work we have not directly identified the epoxide of allylisopropylacetamide as a monooxygenase product, but it is the overwhelmingly likely choice, especially given our gas chromatographic detection of radioactive y-hydroxy-$\delta$-lactone and $\delta$-hydroxy-y-lactone as isolated metabolites after acid-induced lactonization. Both the epoxide and the diol of allylisopropylacetamide will cyclize under these conditions (14).

Is the epoxide of allylisopropylacetamide a sufficiently activated compound to be deleteriously reactive? Ortiz de Montellano and Mico have clearly ruled out the epoxide as the enzyme-bound heme alkylating species in the case of the related allylisopropylmethylster-induced P-450 suicide (11) in which synthetic epoxide was shown clearly to bind to microsomes by P-450 spectral perturbation but not to inactivate. This demonstrated nicely that epoxide as a bound prod-
Suicidal Processing of Allylisopropylacetamide by Rat P-450

The metabolite question then devolves to the fate of the 200 epoxide product molecules released from the phenobarbital-induced cytochrome P-450 active site of the isozymes. Until reactivity studies with synthetic epoxide provide calibration for SN1 reactivity, it may be premature to speculate how deleteriously electrophilic a molecule it represents. However, Smith's detection of the γ-lactone as a principal urinary metabolite (15) suggests the intramolecular capture pathway noted below may be occurring and be one index of epoxide reactivity.

An initial alternate experimental approach is to examine [14C]allylisopropylacetamide-derived radioactivity in cellular macromolecules. Smith (15) noted about 3 nmol of [14C]-label/mg of liver protein and our microsomal incubations show that the fast phase of allylisopropylacetamide-processing yields 1.5 nmol of acid-stable [14C]-label/mg of microsomal protein. With the provisional assumption that an average subunit molecular weight of a microsomal protein is 50,000, this would correspond to 1.5 nmol/20 nmol of protein or, on average, 7.5 of every 100 protein molecules labeled after 200 turnovers of allylisopropylacetamide and inactivation of every susceptible P-450 isozyme molecule in rat liver microsomes. This is one measure of epoxide reactivity. Another perspective is that protein labeling in microsomes accounts for about 4% as much as the soluble metabolites measured. That is, our partition ratios should be corrected to about 210 turnovers per inactivation event, with 8 to 9 product molecules becoming covalently bound to protein. An accounting for allylisopropylacetamide processing by the susceptible P-450 isozyme is presented in Scheme 4.

When this protein-labeling stoichiometry is repeated in the 1:1 reductase/monoxygenase reconstituted system, the labeling comes out 0.67 nmol/mg. Doubling this value to factor out the concomitant uncoupled autoinactivation noted above brings this number to 1.4 nmol/mg, about the same value seen in microsomes. The flavoprotein reductase has a $M_1 = 75,000$, the N-demethylase P-450 isozyme, 50,000. At 1:1 weight concentrations there are 7 nmol of reductase and 10 nmol of P-450/mg of protein. We have not yet analyzed radioactivity localization on gels, but even if it were all on the P-450 enzyme, the [14C]-label would be only 0.15 nmol/nmol of subunit. Apoprotein alkylation from nascently released epoxide product cannot account for a significant amount of observed enzyme inactivation.

An alternate cellular macromolecule as alkylation target is, of course, DNA. We have not yet addressed the question of radioactive alkylation of rat liver DNA by [14C]allylisopropylacetamide but have instead undertaken the initial step of adding exogenous calf thymus DNA as a model DNA. Any results from these in vitro experiments are thus not necessarily meaningful for the cellular DNA situation. They merely use DNA as a chemical sponge and ask if a reactive electrophile is generated that can diffuse to the DNA and alkylate it. By that narrow experimental criterion, the putative allylisopropylacetamide epoxide is also a reasonable alkylating agent. The characterization of adducted DNA is as yet preliminary, but the associated radioactivity fulfills standard purification criteria for covalent labels which have previously been applied for metabolically activated DNA labels (24, 28).

At a level of 1 adduct/4-5000 nucleotides (2500 base pairs) from typical microsomal incubations, the labeling is about 20-fold over background and prevented by SKF-525-A or by preincubation with unlabeled allylisopropylacetamide with the kinetics expected for susceptible P-450 isozyme inactivation. Under equivalent incubation conditions such molecules

\[ \text{HEME ADDUCT} \]

\[ \text{PROTEIN} \]

\[ \text{DNA} \]

\[ \text{METABOLITES} \]
as aflatoxin and benzo-[a]-pyrene give about equivalent amounts of DNA adducts. On a quantitative basis, the percentage of label in DNA compared to that recoverable as allylisopropylacetamide-products suggests about 1 product molecule in 1000 was intercepted by exogenous calf thymus DNA. That is, for every 5 molecules of P-450 isozyme inactivated by allylisopropylacetamide, 1 adduct/DNA is made. It is of interest to determine if this level of adduct occurs in rat liver DNA and whether it has any pathological consequences compared to the acute hepatotoxic response from P-450 heme alkylation. In this connection we find that allylisopropylacetamide is not mutagenic in human cell forward mutation assays (29), suggesting rat liver DNA is not a significant target.

It remains to be seen whether partition ratios for allylisopropylacetamide are constant from one susceptible isozyme to another within a given cell, tissue, or between organisms, and whether they correlate with differential toxicity. Since the number of turnovers per inactivation is a sensitive kinetic index of even subtly different steric placements in a molecule in 1000 was intercepted by exogenous calf thymus DNA, it may be relevant to compare the kinetic parameters. The Nucleic Acids: Chemistry and Biology (Chargriff, E., and Davidson, J. N. eds), Vol. 2, Academic Press, New York.

After submission of this manuscript, a report has appeared from Mico and Ortiz de Montellano (30) indicating about the same partition ratio (220:1) for allylisopropylacetamide suicidal processing in microsomes.

REFERENCES