Lipid peroxidation in biological membranes is known to yield reactive aldehydes, of which trans-4-hydroxy-2-nonenal (HNE) is particularly cytotoxic. This laboratory previously reported that purified liver microsomal P450 cytochromes are directly inactivated to varying extents by HNE. We have now found a mechanism-based reaction in which P450s are inactivated by HNE in the presence of molecular oxygen, NADPH, and NADPH-cytochrome P450 reductase. The sensitivity of the various isozymes in the two pathways is different as follows: P450 2B4 and the orthologous 2B1 are inactivated to the greatest extent and 2C3, 1A2, 2E1, and 1A1 to a somewhat lesser extent by the pathway in which HNE undergoes metabolic activation. In contrast, 2B4 and 2B1 are insensitive to direct inactivation, and the reductase is unaffected by HNE by either route. Recent studies on the catalytic activities of the T302A mutant of P450 2B4 have shown that the rate of oxidation of a variety of xenobiotic aldehydes to carboxylic acids is decreased, but the rates of aldehyde deformylation and mechanism-based inactivation of the cytochrome are stimulated over those of the wild-type enzyme (Raner, G. M., Vaz, A. D. N., and Coon, M. J. (1997) Biochemistry 36, 4895–4902). Inactivation by those aldehydes apparently occurs by homolytic cleavage of a peroxyhemiacetal intermediate to yield formate and an alkyl radical that reacts with the heme. In sharp contrast, the rate of mechanism-based inactivation by HNE is decreased with the T302A mutant relative to that of the wild-type P450 2B4, and mass spectral analysis of the heme adduct formed shows that deformylation does not occur. We therefore propose that the metabolic activation of HNE involves formation of an acyl carbon radical that leads to the carboxylic acid or alternatively reacts with the heme.

The toxicity of lipid peroxidation products generated in biological membranes is widely attributed to reactive aldehydes, including 2-alkenals and 4-hydroxyalkenals (1). Of these, trans-4-hydroxy-2-nonenal (HNE) is produced in relatively large amounts and is particularly deleterious in that it is cytotoxic (2), lyses erythrocytes (3), and inhibits DNA and protein synthesis (4, 5). Stadtman and colleagues (6–8) have described the inactivation of purified cytosolic enzymes by HNE and have shown the involvement of protein sulphydryl, amino, and imidazole groups. Since the first target of the toxic products of lipid peroxidation would be expected to be membranous enzymes, this laboratory recently undertook a study of the sensitivity of hepatic microsomal P450 (see Ref. 10 for updated P450 nomenclature) cytochromes to HNE (9). The six purified isozymes that were examined were found to differ in the effect of this agent at various concentrations, with P450s 1A1, 2E1, and 1A2 being most readily inactivated.

The present paper is concerned with a newly discovered reaction in which HNE undergoes metabolic activation by cytochrome P450 in a reconstituted oxygenating system containing molecular oxygen, NADPH, and the reductase. All of the P450 isozymes examined are inactivated in this mechanism-based reaction, but the pattern is strikingly different from that seen in the earlier studies on the direct effect of HNE, that is, in the absence of NADPH and reductase. For example, P450 2B4, the phenobarbital-inducible isozyme (11), is unaffected by direct exposure to HNE but is the most sensitive of all of the cytochromes examined to metabolically activated HNE.

This laboratory has been interested in the deformylation of aldehydes as a model for the final step in the aromatase-catalyzed reaction in which androgens are converted to estrogens (12) and has shown that similar oxidative cleavages occur with purified liver microsomal P450s. The reactions include the conversion of cyclohexane carboxaldehyde to cyclohexene (13), of a series of low molecular weight branched chain aldehydes to the n = 1 alkenes, and of citronellal to 2,6-dimethyl-1,5-heptadiene (14), and of 3-oxodecan-4-ene-10-carboxaldehyde, a bicyclic steroid analog, to 3-hydroxy-6,7,8,9-tetrahydroxynaphthalene (15). Our recent investigation on the effect of site-directed mutagenesis of threonine 302 to alanine on the activities of recombinant P450 2B4 (16) drew on earlier evidence from others that the corresponding mutation in bacterial P450cam interferes with the activation of dioxygen to the oxenoid species, apparently by disrupting proton delivery to the active site (17, 18). Of particular interest, the deformylation of cyclohexene carboxaldehyde by the mutant 2B4 P450 to produce cyclohexene is greatly increased, which along with other evidence led us to conclude that the iron-peroxy species, rather than the iron-oxene species, is the direct oxygen donor.

More recently, we have examined the mechanism by which a series of xenobiotic aldehydes bring about the mechanism-based inactivation of P450 2B4 (19). The results obtained with the T302A mutant of P450 2B4, along with isotope effects and observations on the influence of aldehyde structure, indicated that an alkyl free radical produced upon decomposition of the.
TABLE I

Direct inactivation of enzymes by HNE

| Enzyme          | Inactivation % |
|-----------------|---------------|
| P450 1A1        | 54 ± 2        |
| P450 1A1 (rat)  | 38 ± 2        |
| P450 2A10/2A11  | 28 ± 1        |
| P450 2E2        | 17 ± 1        |
| P450 1A2        | 16 ± 2        |
| P450 2E1 (human)| 12 ± 1        |
| P450 2E1        | 10 ± 1        |
| P450 2C3        | 11 ± 1        |
| P450 2B4        | 0             |
| P450 3A6        | 0             |
| P450 2B1 (rat)  | 0             |
| P450 2G1        | 0             |
| Reductase       | 0             |

The inactivation of P450 isozymes was determined as described under "Experimental Procedures." The source of the various purified enzymes was rabbit liver microsomes unless otherwise indicated. When the reductase (0.1 nmol) was examined for inactivation by HNE, P450 was omitted from the reaction mixture, and 0.1 nmol of isozyme 2B4 or 2E1 (but no additional reductase) was added to the assay mixture for 1-phenylethanol oxidation.

proposed peroxyhemiacetal intermediate reacts with the heme and thus causes the observed inhibition. In contrast, as described below, our evidence on the nature of the heme adduct formed from HNE points to the involvement of an acyl carbon radical.

EXPERIMENTAL PROCEDURES

Materials—Rabbit liver microsomes served as the source of purified P450 cytochromes 2E1, 2B4, 1A1, 1A2, 2C3, and 3A6 (9, 20–22) and NADPH-cytochrome P450 reductase (23). P450 2G1 was purified from rabbit olfactory microsomes (24) and 2E2 from liver microsomes of neonatal rabbits (25). Recombinant human P450 2E1 (26) was kindly provided by Dr. P. F. Guengerich and L. C. Bell, Vanderbilt University; recombinant rat P450 1A1 by Dr. A. Parkinson, University of Kansas, and Dr. P. F. Hollenberg, The University of Michigan; and recombinant rabbit P450 2A10/11 (27) by Dr. X. Ding, New York State Department of Health. Truncated P450 2B4 (32–27) and the corresponding T302A mutant (16) were prepared and generously provided by Dr. Dermot McGinnity and Dr. Gregory M. Raner. HNE, dimethylacetal, synthesized by the method of Nadkarni and Sayre (28), was hydrolyzed by treatment with 1 mM HCl at room temperature for 30 min. The concentration of the HNE stock solution, which was prepared daily, was determined from the absorbance at 224 nm (29).

Assay for Direct Inactivation of P450 Cytochromes by HNE—A typical reaction mixture contained the following components, added in the order indicated: purified P450 isozyme (0.1 to 0.2 nmol), potassium phosphate buffer, pH 7.4 (12.5 mM), and HNE (40 nmol) in a final volume of 0.20 ml. After incubation for 15 min at 30 °C, the mixture was placed on ice and promptly assayed for catalytic activity in the oxidation of 1-phenylethanol to acetophenone (30). For this purpose, NADPH-cytochrome P450 reductase (equimolar with respect to the P450), freshly dispersed DLPC (15 μg), 1-phenylethanol (5 μmol), and NADPH (0.5 μmol) were added in a final volume of 0.5 ml. After incubation for 20 min at 30 °C the reaction was stopped with 0.25 ml of 6% perchloric acid, and acetophenone was determined by the method of Nadkarni and Sayre (28). The cytochrome was exposed to 0.4 mM HNE before residual activity was determined. Inactivation was calculated by comparison of the activity with and without HNE present.

Assay for Mechanism-based Inactivation of P450 Cytochromes by HNE—The reaction mixture contained a purified P450 isozyme (1.0 nmol), reductase (1.0 nmol), DLPC (20 μg), catalase (100 units), superoxide dismutase (100 units), potassium phosphate buffer, pH 7.4 (17.5 mM), HNE (140 nmol), and NADPH (0.35 μmol) in a final volume of 0.35 ml. The reaction was at 30 °C, and a 10-μl aliquot was removed at various time points and added to a solution containing 1-phenylethanol (5 μmol), DLPC (6 μg), phosphate buffer, pH 7.4 (50 mM), and NADPH (0.5 μmol) in a total volume of 0.5 ml. Acetophenone was quantitated by HPLC, and the percentage inhibition was ascertained by comparison of the activity with and without HNE present.

Determination of Heme and Detection of Heme Adducts—To a solution containing P450 2B4 (1.0 nmol), reductase (1.0 nmol), DLPC (40 μg), phosphate buffer, pH 7.4 (50 mM), and HNE (400 nmol), NADPH (1.0 μmol) was added to initiate the reaction in a final volume of 1.0 ml. The mixture was incubated at 30 °C for 20 min and analyzed by HPLC at the flow rate of 1.0 ml/min. A solvent linear gradient was programmed to deliver acetanilide, aqueous 0.1% trifluoroacetic acid from a 40:60 to a 50:50 ratio over 15 min, then to 80:20 over 15 min, and finally to 100% acetanilide over 5 min. Heme and heme adducts were detected by a variation of the procedure described by Osawa et al. (31, 32) in studies on myoglobin. A Waters 996 photodiode array detector was used connected to the HPLC. The native heme, heme adduct 1, and heme adduct II (a very small, unidentified peak) had retention times of 12.15, and 30 min with absorption maxima of 398, 403, and 413 nm, respectively. Electrospray and MALDI mass spectral analyses were performed at The University of Michigan mass spectrometry core facility with a VG Fisons "Platform" single quadrupole mass spectrometer and Perspective Biosystems Vestec Lasertec MALDI Linear Time-of-Flight Mass Spectrometer with the laser set at 327 nm. Samples were introduced in 50:50:0.05 acetoni trile:H2O:trifluoroacetic acid.
RESULTS

Direct Inactivation of Purified Microsomal Enzymes by HNE—Individual P450 isozymes were incubated with 200 μM HNE for 15 min as described under "Experimental Procedures" and subsequently assessed for catalytic activity with 1-phenylethanol in the oxidative conversion to acetophenone. This is a useful assay because all of the isozymes so far examined catalyze the reaction. The results given in Table I show that P450 1A1 is by far the most sensitive to inactivation by HNE, followed by 2A10/2A11 (a mixture of two isoforms differing in only eight amino residues), with 2E2, 1A2, 2E1, and 2C3 being only moderately sensitive. Several other isoforms, including 2B4, undergo negligible loss of activity. In our earlier paper by Bestervelt et al. (9), somewhat different relative losses in activity of the various isozymes were observed, and the results were found to depend on the HNE concentrations used. At that time we were unaware of the occurrence of the mechanism-based inactivation to be described in this paper. The experimental conditions under which the P450 cytochromes were exposed to HNE differ in several respects from those used previously, and the aldehyde was generated from the dimethylacetal by 1 mM rather than 1 M HCl. The stronger acid used in our earlier studies may have led to the formation of additional inhibitory products.

The reductase was also exposed to HNE and subsequently assayed for catalytic activity upon supplementation with P450 2B4 or 2E1. No inactivation was detected (Table I), which provides assurance that the results with the individual P450 isozymes are not attributable to impairment in the ability of the flavoprotein to transfer electrons from NADPH to the cytochromes. In experiments not presented in which several of the P450 isozymes were individually exposed to HNE, similar results were obtained whether at the end of the reaction the mixtures were diluted 50-fold or dialyzed overnight against 1 liter of 50 mM phosphate buffer, pH 7.4, containing 10% glycerol, before catalytic activity was determined. Therefore, the results in Table I are not attributable to a contribution of HNE to the mechanism-based inactivation to be described below.

The extent of direct inactivation is linear with respect to time, as shown with P450 1A2 in Fig. 1, but the reaction does not go to completion. The inactivation was examined at 20 min as a function of the HNE concentration. The results in Fig. 2 indicate that the impairment in catalytic competence correlates well with the decrease in CO-reactive P450. In additional ex-

| Isoform        | Inactivation |
|---------------|-------------|
| P450 2B4      | 57 ± 9      |
| P450 2B1 (rat) | 47 ± 6      |
| P450 2E3      | 33 ± 6      |
| P450 1A2      | 32 ± 6      |
| P450 2E1      | 30 ± 3      |
| P450 1A1 (rat)| 30 ± 9      |
| P450 2E1 (rat)| 27 ± 6      |
| Reductase     | 2 ± 0       |

Bestervelt et al. (9), somewhat different relative losses in activity of the various isozymes were observed, and the results were found to depend on the HNE concentrations used. At that time we were unaware of the occurrence of the mechanism-based inactivation to be described in this paper. The experimental conditions under which the P450 cytochromes were exposed to HNE differ in several respects from those used previously, and the aldehyde was generated from the dimethylacetal by 1 mM rather than 1 M HCl. The stronger acid used in our earlier studies may have led to the formation of additional inhibitory products.

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![Graph](image1.png)

![Graph](image2.png)

![Graph](image3.png)

![Graph](image4.png)
experiments not presented, the typical 29% decrease in P450 1A2 treated with 400 μM HNE for 20 min resulted in only a 6% increase in P420, but the total heme as determined by HPLC remained unchanged. Furthermore, no evidence could be obtained by HPLC for the formation of a modified heme.

**Mechanism-based Inactivation of Purified Microsomal Enzymes by HNE—**The NADPH-dependent metabolic activation of HNE was discovered with P450 isozyme 2B4, which, as described above, is unaffected by this agent in the absence of the reduced pyridine nucleotide. The requirements for this reaction are shown in Table II. The usual components of the functional reconstituted enzyme system in addition to P450 are required, including the reductase and molecular oxygen as well as NADPH. Catalase and superoxide dismutase are routinely included to avoid possible inactivation of P450s by hydrogen peroxide, superoxide, and other reactive oxygen species formed in the complete system, but their omission was without effect on the results with isozyme 2B4. This and other isoforms were examined as shown in Table III. 2B4 and the orthologous rat cytochrome, 2B1, are highly inactivated, and 2C3, 2E1, and 1A2 are more sensitive than in the direct (NADPH-independent) inactivation. In contrast, 1A1, which is the most sensitive to direct exposure to HNE, is inactivated to a lesser extent in the mechanism-based reaction. This was determined by including a control experiment in which NADPH was omitted and making the necessary correction. The reductase was also examined for possible mechanism-based inactivation. After incubation of the complete system containing P450 2B4 with HNE, the rate of 1-phenylethanol oxidation was determined in the presence of additional 2B4 but no reductase. Under these conditions reductase inactivation was negligible, thus providing assurance that the results with the various P450s in Table III are due to an effect on the cytochromes and not to interference with electron transfer from NADPH because of inhibition of the flavoprotein.

**Kinetics of Mechanism-based Reaction—**The time course of inactivation of the six P450 isoforms by 0.4 mM HNE in the complete reconstituted enzyme system is shown in Fig. 3. A time-dependent decline in catalytic activity in 1-phenylethanol oxidation was observed with all of the P450s investigated, albeit at different rates; inactivation at 20 min was about 30% for human or rabbit 2E1 and rat 1A1, and 50 to 60% for the 2B1 and 2B4 orthologues. Isoforms 1A1 and 1A2 exhibit both direct and mechanism-based inactivation, and the rates required correction for those determined in the absence of NADPH, as shown in Fig. 4. The effect of inactivator concentration on P450 2B4 was also determined, as indicated in Fig. 5. Inactivation increased with time in an apparent biphasic manner. Biphasic kinetics were also observed with 7-ethoxy-4-trifluoromethylcoumarin as substrate. From double-reciprocal plots of such kinetic data, the apparent Kᵢ and k inactivation were found to be 0.31 mM and 0.076/min, respectively, for the fast phase of the reaction (0–5 min). The corresponding values for the slow phase (10–20 min) are 0.75 mM and 0.02/min. The partition coefficients were also determined from the kinetic data. The values are about 80 and 70 for the fast and slow phases, respectively. Coefficients of this magnitude suggest that the activated HNE intermediate that inactivates P450 has at least one other metabolic fate.

With purified P450 2B4 in a reconstituted system, a heme adduct was generated during mechanism-based inactivation by
P450 Inhibition by Metabolic Activation of 4-Hydroxy-2-nonenal

The scheme in Fig. 8 summarizes our present knowledge of inactivation of xenobiotic aldehydes with involvement of the P450 peroxo-iron species resulting in deformylation and production of an alkyl radical that yields the $n-1$ olefin and alcohol or gives the heme adduct.

Whereas the T302A mutant of truncated P450 2B4 is more active than the corresponding wild-type cytochrome in the inactivation by xenobiotic aldehydes (19), the reverse was found to be true with HNE. As shown in Fig. 7, the mutant protein is about 30% less effective in inactivation. In results not presented, the 4-hydroxy-2-nonoic acid formed under the same conditions was identified by HPLC according to Hartley et al. (33); the mutant protein is about 34% less effective in this conversion. These findings, along with the molecular weight of the heme adduct, support our idea that P450 inactivation by HNE involves formation of a radical species associated with oxidation to the carboxylic acid rather than with loss of the aldehyde carbon by the deformylation route.

**DISCUSSION**

The results presented establish a number of differences between the direct inhibition of P450 cytochromes by HNE described earlier (9) and the inhibition requiring metabolic activation. The latter reaction has a clear requirement for the presence of NADPH, molecular oxygen, and the reductase, thus providing a reconstituted system in which HNE undergoes oxidative mechanism-based activation. The isozyme specificity shows some similarity but also some striking differences. Whereas direct exposure to HNE has no effect on rabbit P450 2B4 or the orthologous rat 2B1, these cytochromes are by far the most sensitive of those examined to metabolically activated HNE. The reverse is true of P450 1A1, which is the most sensitive of the isozymes to direct inactivation but only moderately affected in the mechanism-based reaction. Several of the cytochromes are somewhat inactivated by both routes.

Mechanistically the reactions are also quite different. No heme adducts could be detected upon direct exposure of P450s to HNE, suggesting that a reaction occurs instead with reactive amino acid residues in the protein. In contrast, the metabolic activation of HNE in the mechanism-based reaction described in the present paper leads to the formation of a heme adduct. The scheme in Fig. 8 summarizes our present knowledge of P450 inactivation by aldehydes in the reconstituted oxygenating system and shows the requirement for protons in the active site for cleavage of the oxygen-oxygen bond in the oxenoid pathway but not for the pathway in which peroxo-iron is the direct oxygen donor. In the case of HNE, hydrogen abstraction yields the carboxyl carbon radical, which combines with the iron-bound hydroxyl radical by the widely accepted "oxenoid" mechanism (34–38) to give the carboxylic acid. Alternatively, the radical attacks the heme to give the adduct in which the carbon chain of the starting aldehyde remains intact. The 2B4 T302A mutant is less effective in acid formation and in P450 inactivation, apparently because of disruption of proton delivery. In contrast, in the case of the saturated and unsaturated xenobiotic aldehydes studied previously (19), reaction with the iron-peroxo species gives the peroxymethacetal, which upon deformylation yields an alkyl radical that gives the olefin or alcohol with one less carbon atom than in the starting aldehyde. Alternatively, this radical attacks the heme to give the $C_{n-1}$ adduct. The T302A mutation results in enhanced activity in the formation of these products and in inactivation of the cytochrome, as predicted by the scheme in Fig. 8.

Since the P450 cytochromes play a primary role in the metabolism of chemical carcinogens and drugs, as well as in the synthesis of biologically important compounds such as steroids and retinoids, inactivation of these catalysts by the products of lipid peroxidation may be of considerable physiological significance. HNE was selected for our studies because it is known to be particularly damaging in leading to the pathophysiological effects associated with oxidative stress in cells and tissues. However, other products of membrane lipid peroxidation, including alkanals, alkenals, 2,4-alkadienals, and various hydroxylaldehydes, should also be considered as potential inactivators. Although the direct and mechanism-based routes for inhibition by HNE affect a somewhat different set of P450 isozymes, together they inactivate all of the purified cytochromes we have examined.

Hochstein and Ernster (39) were the first to report that lipid peroxidation is coupled to the NADPH oxidase system of microsomes. More recently we showed that P450 2E1, the alcohol-inducible form, and P450 2B4, the phenobarbital-inducible form, are the most active of a series of isozymes examined in the reductive cleavage of fatty acid hydroperoxides to hydrocarbons and aldehyde acids (40). Furthermore, 2E1 is particularly effective in the reduction of molecular oxygen to reactive species that may contribute to the initiation of membrane lipid peroxidation (41). In view of the activities of these two isoforms in particular and also of other P450s in generating lipid hydroperoxides and in their \textit{$\delta$}-scission to aldehydic compounds,
inactivation by HNE as a product of lipid peroxidation may provide a negative regulatory function.

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