\textbf{\textit{p-Nitrophenyl Vinyl Ether, A Novel Substrate for the Assay of Cytochrome P-450 Dependent Olefinic Epoxidation in Hepatic Microsomes}}

MASAKAZU ISobe, TOMOMICHI SONE, AND EIGO TAKABATAKE

Department of Toxicology, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka, 573-01, Japan

(Received November 28, 1984)

\textit{p-Nitrophenyl vinyl ether} (NPVE) was metabolized to \textit{p-nitrophenol} and glycolaldehyde via an epoxide by rat hepatic microsomes. The cofactor requirement and effects of monoxygenase inhibitors indicated that the oxidative metabolism of NPVE was mediated by microsomal cytochrome P-450. Epoxide hydrolase plays a minor role, because a strong epoxide hydrolase inhibitor, 3,3,3-trichloropropene oxide, showed a weak inhibitory effect on the \textit{p-nitrophenol} formation. The epoxy intermediate is so labile that the hydrolysis of the epoxide proceeds mostly nonenzymically even at a neutral pH. Induction experiments suggested that NPVE was susceptible to a wide variety of cytochrome P-450 species.

Thus, a convenient and sensitive method for the assay of olefinic epoxidase activity in hepatic microsomes was developed with NPVE as a substrate.

\textbf{Keywords} — \textit{p-nitrophenyl vinyl ether}; \textit{1-ethyloxy-4-nitrobenzene}; glycolaldehyde; \textit{p-nitrophenol}; microsomal oxidation; cytochrome P-450; epoxide; colorimetry; olefinic epoxidation; hepatic metabolism

\section*{INTRODUCTION}

Cytochrome P-450 plays important roles in the metabolism of numerous endogenous molecules and xenobiotics such as steroids, drugs, and carcinogens. Although a variety of substrates have been used for the studies on cytochrome P-450, substrates available for convenient routine assay are limited. For example, aminopyrine\textsuperscript{1} and benzphetamine\textsuperscript{2} are used for the assay of \textit{N-dealkylation} activity, \textit{p-nitroanisole},\textsuperscript{3} \textit{p-nitrophenetole},\textsuperscript{4} and ethoxy-coumarin\textsuperscript{5} for \textit{O-dealkylation} activity, and aniline\textsuperscript{6} and coumarin\textsuperscript{7} for aromatic hydroxylation activity. Further, no substrate is there for the spectrophotometric assay of olefinic epoxidation activity. It has been well known that the metabolism of olefinic double bond to the epoxide by cytochrome P-450 is often a critical determinant to the toxic responses and to the metabolic fate of chemicals. Carcinogens and mutagens such as aflatoxin B\textsubscript{1},\textsuperscript{8} vinyl chloride,\textsuperscript{9} trichloroethylene,\textsuperscript{10} acrylonitrile,\textsuperscript{11} and styrene\textsuperscript{12} are all metabolically activated by microsomal cytochrome P-450 to the respective olefinic epoxides which bind covalently to biomacromolecules, deoxyribonucleic acid (DNA) and proteins. But the enzymic bases are not yet unequivocal. Therefore, a convenient method with an olefinic substrate is required for the assay of microsomal P-450.

The present study provides a colorimetric method for the assay of olefinic epoxidation activities by means of \textit{p-nitrophenyl vinyl ether} (NPVE) as the model substrate.

\section*{MATERIALS AND METHODS}

\textbf{Materials} — Nicotinamide adenine dinucleotide phosphate (NADP), NADPH, nicotinamide adenine dinucleotide (NADH), glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Tokyo. Metyrapone and SKF 525-A were donat-
ed from Nippon CIBA-Geigy Co., Tokyo and Smith Kline & French Laboratories, Philadelphia, Pa., respectively. Other reagents used were all reagent grade. \( p \)-Nitrophenyl vinyl ether (1-ethenoyloxy-4-nitrobenzene, NPVE) and glycolaldehyde 2,4-dinitrophenylhydrazone were synthesized according to the methods reported by Dombroski and Hallensleben\(^{19}\) and Honda et al.,\(^{14}\) respectively.

**Microsomal Preparations** — Male Wistar rats weighing 150—160 g were used. Rats received phenobarbital (80 mg/kg body weight) in saline \( i.p. \) for 2 d, 3-methylcholanthrene (50 mg/kg) in corn oil \( i.p. \) once 2 d before, or a polychlorinated biphenyl mixture (KC-400, 500 mg/kg) in corn oil \( i.p. \) once 5 d before were sacrificed. The liver removed was homogenized in three volumes of isotonic KCl solution. The homogenate was centrifuged at 9000 \( \times g \) for 20 min and the supernatant fraction separated was then recentrifuged at 105000 \( \times g \) for 1 h. The resulting pellet was resuspended to give one g equivalent of liver per ml of KCl-Tris buffer (0.15—0.05 M), pH 7.4. Protein and cytochrome P-450 were determined by the method of Lowry et al.,\(^{18}\) and Omura and Sato,\(^{16}\) respectively.

**Assay of Microsomal Oxidation** — Incubations for the assay of microsomal oxidation of NPVE were carried out as follows. NPVE (1 \( \mu \)mol) dissolved in 0.1 M phosphate buffer, pH 7.4, containing 0.01% Tween 80 and 2.0% methanol (2.0 ml) was preincubated at 37 °C for 5 min with the microsomal suspension (1.0 ml) diluted to 0.2—4 mg protein per ml. The enzymic reaction was started by the addition of an NADPH-generating system (2.0 ml) which consist of NADP (2.5 \( \mu \)mol), glucose 6-phosphate (25 \( \mu \)mol), MgCl\(_2\) (25 \( \mu \)mol), glucose 6-phosphate dehydrogenase (5 IU), and 0.1 M phosphate buffer, pH 7.4. The mixture was incubated at 37 °C for 30 min by gentle shaking under aerobic conditions and the enzymic reaction was terminated by the addition of 15% ZnSO\(_4\) solution (2.0 ml). To remove the microsomal protein from the reaction mixture, a saturated \( \text{Ba(OH)}_2 \) aqueous solution (2.0 ml) was added, then the mixture was alkalinized with 5 N NaOH solution (0.5 ml). After centrifugation of the mixture at 3000 rpm for 5 min, \( p \)-nitrophenol in the supernatant was determined by the absorbance at 420 nm with a Shimadzu model UV 3000 spectrophotometer.

Assay of glycolaldehyde formed from NPVE by microsomal oxidation was carried out as follows. The above mentioned microsomal oxidation reaction was terminated by the addition of 2 mM 2,4-dinitrophenylhydrazine dissolved in 5 N \( \text{H}_2\text{SO}_4 \) (1 ml). After standing for 1 h at room temperature, the mixture was extracted with \( \text{C}_6\text{H}_6 \) (10 ml). The residue obtained by evaporation of the solvent from the organic phase was dissolved in \( \text{C}_6\text{H}_6 \) (0.5 ml). The solution was applied onto a silica gel column (4 × 20 mm). The column was washed by \( \text{C}_6\text{H}_6-\text{Me}_2\text{CO} \) (20:1, 2 ml) for removing the excess reagent and then the hydrazone derived from glycolaldehyde was eluted with \( \text{C}_6\text{H}_6-\text{Me}_2\text{CO} \) (1:1, 2 ml). The residue obtained by the evaporation of solvents from the eluate was dissolved in MeOH (0.5 ml) to examine for the content of glycolaldehyde 2,4-dinitrophenylhydrazone by high performance liquid chromatography (HPLC). HPLC was carried out on an octadecylsilicone column (Zorbax ODS, \( 4 \times 250 \) mm) equipped with a ultraviolet (UV) monitor (353 nm). The mobile phase used for the HPLC was MeOH—H\(_2\)O (3:2) at a flow rate of 1.0 ml/min. Under these conditions, 2,4-dinitrophenylhydrazine and authentic glycolaldehyde 2,4-dinitrophenylhydrazone were eluted at 5.8 and 6.9 min, respectively. The amount of glycolaldehyde accumulated in the incubation mixture was determined from the peak area.

Assay of NPVE remained in the incubation mixture was carried out as follows. After the microsomal incubation, the reaction was terminated by the addition of 5 N NaOH solution (0.5 ml) and extracted with \( n \)-hexane (2.0 ml). Aliquots of the extract were examined for the NPVE content by HPLC on a silica column (LiChrosorb SI-60, 5 μ, \( 4 \times 250 \) mm). The mobile phase used for the HPLC was \( n \)-
hexane-tetrahydrofuran (100:1) at a flow rate of 2.0 ml/min. The effluent was monitored at 295 nm. Under these conditions, NPVE was eluted at 4.0 min.

Assay of O-demethylase activity was carried out with p-nitroanisole as the substrate (0.2 mM in the incubation mixture) according to the method for the assay of p-nitrophenol from NPVE.

The side chain oxidation of styrene by microsomes was assayed according to the method of previous report. The rate of side chain oxidation was expressed as a sum of styrene oxide and styrene glycol formed.

RESULTS

Microsomal Oxidation of p-Nitrophenyl Vinyl Ether (NPVE)

NPVE was incubated with the untreated rat hepatic microsomes (4 mg protein) according to the standard method at 37 °C. The reaction mixture was colored yellow during the incubation in the presence of an NADPH-generating system, but not colored when the microsomal suspension or the NADPH-generating system was omitted from the incubation mixture. After deproteinization, the yellowish substance in the reaction mixture was identified to p-nitrophenol by UV spectrophotometry. The deproteinized solution showed a UV absorption maximum at 400 nm and a blue shift by acidifying with 1 N H₂SO₄ as well as authentic p-nitrophenol solution.

Glycolaldehyde, the other product derived from NPVE by the microsomal oxidation, was expected to accumulate in the reaction mixture. To the reaction mixture was added an acidic 2,4-dinitrophenylhydrazine solution and the al-

---

**FIG. 1. Identification of Glycolaldehyde as 2,4-Dinitrophenylhydrazone by High Performance Liquid Chromatography**

HPLC conditions: column, Zorbax ODS (4 x 250 mm); mobile phase, MeOH/H₂O (6:4); flow rate, 1.0 ml/min; monitor, UV at 353 nm. a) extract of the microsomal reaction mixture, b) authentic glycolaldehyde 2,4-dinitrophenylhydrazone, c) authentic 2,4-dinitrophenylhydrazone.

---

**TABLE I. Formation of p-Nitrophenol and Glycolaldehyde from p-Nitrophenyl Vinyl Ether by Microsomal Oxidation**

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Substrate recovered (µmol)</th>
<th>Products formed (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p-Nitrophenol</td>
</tr>
<tr>
<td>0</td>
<td>0.987 ± 0.012</td>
<td>0.005 ± 0.003</td>
</tr>
<tr>
<td>10</td>
<td>0.680 ± 0.004</td>
<td>0.344 ± 0.010</td>
</tr>
<tr>
<td>20</td>
<td>0.539 ± 0.004</td>
<td>0.490 ± 0.009</td>
</tr>
<tr>
<td>80</td>
<td>0.111 ± 0.003</td>
<td>0.872 ± 0.009</td>
</tr>
</tbody>
</table>

Incubation mixture contained the hepatic microsomal preparation (3.2 mg protein/ml) from untreated rat, p-nitrophenyl vinyl ether (0.2 mM), NADP (0.5 mM), glucose 6-phosphate (5 mM), MgCl₂ (5 mM), and glucose 6-phosphate dehydrogenase (1 IU/ml) in 5 ml of 0.1 mM, phosphate buffer, pH 7.4. The incubation was carried out at 37 °C.

The results represent the mean ± S.E. of three incubations.
dehyde was derivatized to the hydrazone. The hydrazone fraction extracted from the incubation mixture with C₆H₆ was examined by HPLC. The chromatogram showed a major peak which had the same retention time as that of authentic glycolaldehyde 2,4-dinitrophenylhydrazone (Fig. 1). The component of the major peak was isolated and identified to glycolaldehyde 2,4-dinitrophenylhydrazone by UV spectrophotometry (λ<sub>max</sub> 353 nm) and mass spectrometry (M<sup>+</sup>, m/z = 240). It has also become evident that other minor peaks in the chromatogram are originated from microsomal components or incubation ingredients because zero time incubation mixture showed small peaks with the same retention times in the chromatogram.

*p*-Nitrophenol and glycolaldehyde formed during the microsomal incubation of NPVE were assayed. As the results, a stoichiometric relationship between *p*-nitrophenol and glycolaldehyde formation was observed (Table I). Further microsomal metabolism of both *p*-nitrophenol and glycolaldehyde was also studied. Both metabolites added to the standard incubation mixture were recovered without significant decrease after the microsomal incubation at 37 °C for 30 min in the presence of an NADPH-generating system. These results showed that the amounts of both metabolites in the reaction mixture reflect the rate of microsomal epoxidation of NPVE. Under the standard assay conditions the rate of *p*-nitrophenol formation was a linear function of protein concentration over a range from 0.5 to 7 mg microsomal protein per 5 ml of incubation mixture. The time course study indicated that the *p*-nitrophenol formation proceeded linearly for 30 min unless *p*-nitrophenol concentration in the reaction mixture exceeded 25% of the substrate concentration.

**Cofactor Requirement**

Cofactor requirement for the oxidative formation of *p*-nitrophenol was studied with the hepatic microsomes obtained from the untreated rat. Omitting NADP from the NADPH-

<table>
<thead>
<tr>
<th>TABLE II.</th>
<th>Effects of Inhibitors of Monoxygenase and Epoxide Hydrolase on the <em>p</em>-Nitrophenol Formation from <em>p</em>-Nitrophenyl Vinyl Ether by Hepatic Microsomes Obtained from Untreated Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemicals</strong></td>
<td><strong>Concentration</strong></td>
</tr>
<tr>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td>SKF 525-A</td>
<td>0.10 mM</td>
</tr>
<tr>
<td></td>
<td>0.01 mM</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>0.10 mM</td>
</tr>
<tr>
<td></td>
<td>0.01 mM</td>
</tr>
<tr>
<td>7,8-Benzoflavone</td>
<td>0.10 mM</td>
</tr>
<tr>
<td></td>
<td>0.01 mM</td>
</tr>
<tr>
<td>3,3,3-Trichloro-</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>propene oxide</td>
<td>1.0 mM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.2 mM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>N₂/O₂ = 16/1</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>CO/O₂ = 16/1</td>
</tr>
</tbody>
</table>

The microsomal suspension (0.5 mg protein/ml) containing the substrate (0.2 mM), inhibitor, and an NADPH-generating system was incubated at 37 °C for 30 min. a) The enzymic reaction was carried out in a cuvette and *p*-nitrophenol formed during the incubation was continuously monitored with a spectrophotometer.

The results represent the mean ± S.E. of three experiments.
generating system, the formation of p-nitrophenol was not observed. When NADH (1 mM), NADPH (1 mM), or both NADH and NADPH (1 mM each) were used for the microsomal incubation instead of the NADPH-generating system, p-nitrophenol formed at the rates of 0.14, 1.11, or 1.48 nmol/mg microsomal protein/min, respectively. These results showed an absolute requirement of NADPH for the microsomal oxidation as a cofactor. NADH was almost ineffective as an electron donor but gave some synergic effect when added together with NADPH.

**Effects of Inhibitors**

To further characterize the microsomal metabolism, we have examined the effect of some inhibitors on the p-nitrophenol formation (Table II). Well known inhibitor of cytochrome P-450 was added to the microsomal suspension obtained from the liver of untreated rat and the enzymic reaction was carried out. Although SKF 525-A (0.1 mM) reduced the p-nitrophenol formation to 43% of the control, metyrapone (0.1 mM) and 7,8-benzoflavone (0.1 mM) showed weak inhibitory effects. Carbon monoxide, a strong ligand for the reduced heme, inhibited the microsomal formation of p-nitrophenol to 31% of the control by the addition to the incubation atmosphere.

Effect of a potent inhibitor of microsomal epoxide hydrolase, 3,3,3-trichloropropene oxide, was also examined to account for the participation of an epoxy intermediate. We carried out the microsomal incubation in a cuvette at 37 °C monitoring the absorbance at 420 nm continuously. As the result, it was found that 3,3,3-trichloropropene oxide inhibited the p-nitrophenol formation to about a half of the control. On the other hand, the inhibitor reduced only slightly the apparent formation of p-nitrophenol as far as the assay was carried out after deprenization under acidic conditions according to the standard method described in the text.

**Effects of Monoxygenase Inducers**

To define the substrate selectivity of the microsomal monoxygenases, comparative study

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Protein (mg/g liver)</th>
<th>Cytochrome P-450 (nmol/mg protein)</th>
<th>Rates of microsomal oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NPVE (nmol/mg protein/min)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>25.6 ± 0.9 (100)</td>
<td>0.80 ± 0.01 (100)</td>
<td>1.56 ± 0.05 (100)</td>
</tr>
<tr>
<td>3-MC</td>
<td>22.6 ± 0.7 (88)</td>
<td>1.33 ± 0.02 (166)</td>
<td>1.89 ± 0.04 (121)</td>
</tr>
<tr>
<td>PB</td>
<td>28.8 ± 1.0 (113)</td>
<td>2.60 ± 0.26 (325)</td>
<td>3.34 ± 0.27 (214)</td>
</tr>
<tr>
<td>PCB</td>
<td>31.9 ± 1.4 (124)</td>
<td>3.00 ± 0.29 (375)</td>
<td>5.06 ± 0.20 (324)</td>
</tr>
</tbody>
</table>

The hepatic microsomes prepared from male Wistar rats pretreated with 3-methylcholanthrene (3-MC), phenobarbital (PB), or a polychlorinated biphenyl mixture (PCB) were incubated at 37 °C for 30 min with the substrate in the presence of an NADPH-generating system. Substrate concentrations in the incubation mixture were 0.2, 2.0, and 0.2 mM for p-nitrophenyl vinyl ether (NPVE), styrene, and p-nitroanisole, respectively. The results were represent the mean ± S.E. of five animals. Values in parentheses represent % of control.
was carried out with NPVE, styrene, and p-nitroanisole as the substrates. Liver microsomes obtained from rats which had been treated with some typical mono-oxygenase inducers were used. The pretreatment of rats with 3-methylcholanthrene (3-MC), phenobarbital (PB), and a polychlorinated biphenyl mixture (PCB) led to about 1.7-, 3.3-, and 3.8-fold increase in microsomal cytochrome P-450 content per mg protein, respectively. The rates of p-nitrophenol formation from NPVE per mg microsomal protein also increased 1.2-, 2.1-, and 3.2-fold of the control by pretreatment of 3-MC, PB, and PCB, respectively, whereas the rates per cytochrome P-450 did not increase but decrease by pretreatments. These induction profiles for the microsomal oxidation of NPVE were similar to those for the side-chain oxidation of styrene in contrast to those for the O-demethylation of p-nitroanisole (Table III).

Kinetic experiments were carried out with the four different microsomal preparations. The Lineweaver-Burk plots were linear with the substrate concentration over a range from 20 to 200 μM. The apparent $K_m$ of microsomes from control and 3-MC-, PB-, and PCB-pretreated rats was 31, 36, 13, and 19 μM, respectively.

**DISCUSSION**

p-Nitrophenyl vinyl ether (NPVE) yielded equimolar p-nitrophenol and glycolaldehyde by microsomal incubation in the presence of an NADPH-generating system. The metabolic route has been established as illustrated in Fig. 2. It was evident that microsomal cytochrome P-450 involved in the first step reaction, oxidation of NPVE to the epoxide, because the p-nitrophenol formation required NADPH and was inhibited by SKF 525-A and carbon monoxide. The intermediacy of the epoxide was also confirmed by the inhibition experiment. A potent inhibitor of microsomal epoxide hydrolase, 3,3,3-trichloropropene oxide, was found to reduce the p-nitrophenol formation to one half of the control when the reaction is continuously monitored by the absorbance change in a cuvette. However, no significant inhibition of the microsomal p-nitrophenol formation was observed when the assay was carried out after termination of the reaction according to the standard method. The instability of the epoxide reasonably interpreted these observations. Even if the microsomal epoxide hydrolase is completely inhibited by 3,3,3-trichloropropene oxide, the hydrolysis of the labile epoxide proceeds nonenzymatically. As the results, 3,3,3-trichloropropene oxide causes only a slight accumulation of the epoxide and a partial inhibition of the p-nitrophenol formation. It should be the reason for no obvious inhibitory effect of 3,3,3-trichloropropene oxide observed by the standard assay method that the epoxide partially accumulated was nonenzymatically hydrolyzed completely to p-nitrophenol and glycolaldehyde during the processes of termination and deproteinization. The epoxide was also assumed to be unstable by the following facts. Since attempts to detect the epoxide from the biological incubation mixtures did not succeed, we tried to synthesize the epoxide chemically. Epoxidation of NPVE with perbenzoic acid in the absolute CDCl$_3$ yielded the epoxide as far as judged by nuclear magnetic resonance (NMR) spectrometry. But the epoxide could not be isolated from the reaction mixture because the estimated epoxy substance had decomposed with the release of p-nitrophenol under aqueous conditions for the purification. The estimated epoxide decomposed within a few minutes in 0.1 M phosphate buffer, pH 7.4, at 37 °C.

The glycol as the hydrolysis product of the epoxide would be unstable, because it has an electron withdrawing substituent, p-nitrophenoxyl residue, attached to the carbon atom. Involvement of similar glycols in the microsomal metabolism of vinyl chloride, trichloroethylene, acrylonitrile, and hexobarbital has been supposed. All these glycols having an electron withdrawing substituent could not be isolated but yielded degradation products. It is evident that the glycol derived from NPVE by the microsomal metabolism also undergoes rapid nonenzymic degradation to glycolal-
dehydrate and \textit{p}-nitrophenol. Consequently, the amount of \textit{p}-nitrophenol formed is coincident with that of epoxide formed during the microsomal incubation of NPVE. Thus, the assay of \textit{p}-nitrophenol formation could be utilized as a method for the determination of an olefinic epoxidation in microsomes.

The olefinic epoxidation activity in hepatic microsomes was induced by the pretreatment of rats with 3-MC, PB, and PCB. The induction ratios of the NPVE oxidation rate per mg microsomal protein were 1.2-2.1, and 3.2-fold of the control for microsomes obtained from 3-MC-, PB-, and PCB-pretreated rats, respectively. The induction profiles were similar to those of styrene side chain oxidation, a typical olefinic epoxidation, but not to those of \textit{p}-nitroanisole \textit{O}-dealkylation. These profiles showed that NPVE may be utilized as the model substrate for olefinic epoxidation. Although pretreatments of rats by monooxygenase inducers increased the microsomal oxidation per mg protein, they caused some decrease in the oxidation rates of NPVE per molar cytochrome P-450. These results indicate that specific activity for the epoxidation of NPVE is higher in cytochrome P-450(s) of untreated rat liver than in the major cytochrome P-450 species induced by 3-MC-, PB-, or PCB-pretreatment, but the NPVE is susceptible to a wide varieties of cytochrome P-450 species.

Although a wide variety of olefinic chemicals that yield epoxy intermediates by microsomal oxidation have been used for toxicological studies, there is no substrate for the routine assay of olefinic epoxidation activity. Aldrin epoxidation has been reported\cite{20} as a highly sensitive indicator for a microsomal monooxygenase activity, but the assay method is not appropriate for the routine assay because it requires electron capture gas chromatographic analysis. Then, we introduced a convenient and sensitive colorimetric

\[ \text{NPVE} \xrightarrow{\text{Ms P-450}} \text{epoxide} \xrightarrow{\text{H}_2\text{O}} \text{glycol} \]

\[ \text{NP} + \text{CHO} \xrightarrow{\text{non enzymic and enzymic}} \text{GA} \]

\[ \text{NPVE}, \text{p}-\text{nitrophenyl vinyl ether}; \text{NP}, \text{p}-\text{nitrophenol}; \text{GA}, \text{glycolaldehyde}. \]
assay method for the microsomal epoxidation activity with NPVE as the substrate. The substrate concentration of 0.2 mM for the assay was selected, because this concentration was enough higher than $K_m$ values and at the higher concentrations NPVE separated out in crystals. After the deproteinization, $p$-nitrophenol formed from NPVE in the microsomal incubation mixture was determined by the absorbance at 420 nm. The enzymatic reaction proceeded linearly with the amount of microsomes until the oxidation of the substrate reached about 25%. Within the linear region, the olefinic epoxidation activity in microsomes obtained from 20—250 mg of untreated rat liver was assayed. Using a microcuvette for the measurement of absorbance, it is possible to reduce the scale of the incubation volumes less than 1 ml and further increase the sensitivity of the assay by a factor of more than 5. Although this assay method is less sensitive than that of aldrin epoxidation, it is convenient and sensitive enough to determine the olefinic epoxidation as a marker of microsomal monoxygenase activity in comparison with various methods that are widely used for the assay of microsomal marker enzyme activities such as aniline hydroxylase, aminopyrine $N$-demethylase, $p$-nitroanisole $O$-demethylase.

NPVE may be useful not only as the substrate for the assay of microsomal olefinic epoxidation but also as the model compound for toxicological studies about vinyl ethers.

REFERENCES


10) S. Banerjee and B. L. Van Duuren: Covalent binding of the carcinogen trichloroethylene to hepatic microsomal proteins and to exogenous DNA In vitro, Cancer Res., 38, 776—780 (1978).


18) F. Oesch, N. Kaubisch, D. M. Jerina, and J. W. Daly:


