Demethylation of Deuteriated Methoxychlor in Isolated Rat Hepatocytes

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(Received August 16, 1988)

For xenobiotic metabolism studies, isolated rat hepatocytes are considered a better model of in vivo system than liver homogenates or enzyme preparations from liver, because they retain their enzyme systems, cofactors, membranes and various particles related to metabolic reactions.1) We previously studied oxidative demethylation of [dimethyl-\textit{d}_6], [monomethyl-\textit{d}_3], and [\textit{d}_3]-methoxychlor in rat liver microsomes, and reported their isotope effects on the reaction as well as enantiotopic selectivity.2)3) In this paper we describe similar metabolic reactions of the above substrates in rat hepatocytes and some characteristics of the reactions.

MATERIALS AND METHODS

Rat hepatocytes were isolated from male Wistar rats (ca. 200 g, not treated with any inducers) by a collagenase-perfusion method using Krebs-Henseleit balance solution.1) Cells of more than 90% viability were used for all the experiments. Deuteriated methoxychlor analogs (Fig. 1) were prepared by the method previously reported.4) Metabolic reactions using cell suspension (10^6 cells in 2 ml, 37°C) were conducted in the same manner as reported.1,2) Extraction of remaining substrates and metabolites with hexane/ethyl acetate (4:1 v/v), derivatization with pentafluoropropionic anhydride and analysis with a gas chromatograph (electron capture detector, Yanaco G-80E) or with a gas chromatograph-connected mass spectrometer (GC-MS, Shimadzu LKB-9000) were also conducted in the same manner as reported.5,6)

RESULTS AND DISCUSSION

Observed metabolites were mainly monodemethylated and partly di-demethylated products. Table 1 shows the first demethylation rates of methoxychlor at a fixed substrate concentration in rat hepatocytes. In some cases \( V_{\text{max}} \) and \( K_m \) values were obtained from data at various substrate concentrations. The fixed substrate concentration (50 \( \mu \)M) used was relatively high, and the rates at this concentrations in the list are close to the \( V_{\text{max}} \) values of respective substrates. Table 2 shows intramolecular D-H differentiation ratios (apparent intramolecular isotope effects) of respective [\textit{d}_3]-substrates, which indicate that the rat hepatocytes have

1) enantiotopic selectivity of two methoxyl groups of methoxychlor to some degree, and

2) an apparent deuterium isotope effect significantly different from the unity.

It is clear that demethylation in hepatocytes is mainly an oxidative reaction through which the C-H bond in a transition state cleaves and is catalyzed probably by cytochrome \( P-450 \) systems with some enantiotopic selectivity. (The degree

Table 1 Demethylation rates of methoxychlor in rat hepatocytes.

<table>
<thead>
<tr>
<th>Substrate*\textsuperscript{a)</th>
<th>Rate (mol/mol ( P-450 )/min)\textsuperscript{b)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>[\textit{d}_6]-\textit{a})</td>
<td>5.70 \pm 0.21</td>
</tr>
<tr>
<td>(S)-[\textit{d}_3]</td>
<td>5.74 \pm 0.26</td>
</tr>
<tr>
<td>(RS)-[\textit{d}_3]</td>
<td>3.96 \pm 0.04</td>
</tr>
<tr>
<td>(R)-[\textit{d}_3]</td>
<td>2.60 \pm 0.40</td>
</tr>
<tr>
<td>(R)-[\textit{d}_4]</td>
<td>2.67 \pm 0.04</td>
</tr>
</tbody>
</table>

\textsuperscript{a}) Methoxychlor. Initial concentration: 50.0 \( \mu \)M.
\textsuperscript{b}) Disappearance rate of a substrate. Average value \pm SD for two independent preparations of hepatocytes.

\( V_{\text{max}} \): 5.90 mol/mol \( P-450 \)/min. \( K_m \): 3.80 \( \mu \)M.

\( V_{\text{max}} \): 2.75 mol/mol \( P-450 \)/min. \( K_m \): 3.62 \( \mu \)M.

\textsuperscript{c}) Apparent isotope effect value on \( V_{\text{max}} \) = 5.90/2.75 = 2.15.

Fig. 1 Deuteriated methoxychlor analogs.

\[ [\textit{d}_6] : \text{R}_1 = \text{R}_2 = \text{CD}_3, \ \text{R}_1 = \text{CH}_3, \ \text{R}_2 = \text{CD}_3, \]

\[ (R)-[\textit{d}_3] : \text{R}_1 = \text{CD}_3, \ \text{R}_2 = \text{CH}_3. \]
of enantiomeric selectivity of this metabolic reaction cannot be calculated based on the present data only, because the velocity of interconversion of two possible conformers of ES complex involved in the reaction is not determined. For a detailed discussion, see our previous paper.3)

The finding that observed D-H differentiation values of the hepatocytes were smaller than those of microsomes b) (S: 13.94, R: 1.10, RS: 4.88) from untreated rat liver shows a difference in the composition of cytochromes P-450 between hepatocytes and microsomes, suggesting the existence of some cytochrome P-450 components with a lower D-H differentiation capability and a higher catalytic capability in hepatocytes, which might have been partly lost or damaged in microsomes during the preparation. Higher reaction rates in hepatocytes (e.g. \( V_{\text{max}} \)) for \([d_3]\)methoxychlor: 5.90 in hepatocytes versus 1.15 in microsomes b) from untreated rats) support this suggestion. We have also mentioned in the previous report, based on the reaction of aldrin epoxidation, that part of cytochrome P-450 systems would be lost and/or damaged while microsomes are prepared.1) McLean & Day also report that the amount of cytochrome P-450 recovered in a microsomal fraction varies with the amount originally present in liver homogenates.5)

In the hepatocyte experiments, we sometimes observed a significant amount of di-demethylated metabolites (up to 3 to 5% of the monodemethylated metabolites in case of (S)-\([d_3]\)-methoxychlor), formation of which may also have contributed to alter the values of D-H differentiation ratios, because we obtained the ratios from the amount ratios of \(d_3\) and \(d_0\)-counterpart of mono-demethylated metabolites that were present in the reaction mixture. Even if the formation of the di-demethylated metabolites had been taken into consideration to correct the ratio values, however, the intramolecular ratio of the (S)-\([d_3]\)-analog (4.37 in Table 2) would not have increased but only decreased, because the (R)-

\([d_3]\)monomethoxy-monohydroxy compound was demethylated faster than the (S)-\([d_3]\)-isomer (Table 3), and must have been also much faster than the (S)-\([d_3]\)monomethoxy-monohydroxy compound as shown in the Scheme 1. This relation should have reduced the (R)-\([d_3]\)monomethoxy-monohydroxy compound in amount to

Table 2 Intramolecular D-H differentiation ratio of \([d_3]\)methoxychlor analogs in rat hepatocytes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Intramolecular ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S)-([d_3])</td>
<td>4.37 ± 0.59</td>
</tr>
<tr>
<td>(RS)-([d_3])</td>
<td>2.98 ± 0.14</td>
</tr>
<tr>
<td>(R)-([d_3])</td>
<td>0.36 ± 0.02</td>
</tr>
</tbody>
</table>

a) Methoxychlor. Initial concentration: 50.0 \(\mu\)M.
b) The ratio of (monodemethylated [d_3]product) to (monodemethylated [d_0]product). Obtained by a selected ion-monitoring method after pentafluoropropionylation. Average value ± SD for two independent preparations of hepatocytes.

Table 3 Demethylation rates of the monomethoxy-monohydroxy compound (mono-demethylated methoxychlor) in rat hepatocytes and in rat liver microsomes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate (mol/mol P-450/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hepatocytes</td>
</tr>
<tr>
<td>(R)-([d_3])</td>
<td>2.24</td>
</tr>
<tr>
<td>(RS)-([d_3])</td>
<td>1.79</td>
</tr>
<tr>
<td>(S)-([d_3])</td>
<td>1.48</td>
</tr>
</tbody>
</table>

a) Mono-demethylated methoxychlor. The initial substrate concentration was 50.0 \(\mu\)M. Product was di-demethylated methoxychlor.
b) Average value of triplicate runs with one preparation of hepatocytes or microsomes.
c) For (RS)-\([d_3]\)-analog, the rate was 0.55, and the apparent isotope effect was 1.79/0.55 (= 3.26).

Scheme 1 Relative rates of demethylation.
a larger extent than the (S)-[$d_3$]-counterpart, and thus must have changed the observed D-H differentiation ratio at the first step of reaction to a larger value than the true ratio. Therefore, the above suggestion that there are some more cytochromes P-450 with a lower D-H differentiation capability in hepatocytes generally holds.

In this report, we have demonstrated a usefulness of isolated rat hepatocytes in a study on xenobiotic metabolism with enantiotopic selectivity and isotope effects.

REFERENCES

要約
ラットの分離肝細胞による重水素化メトキシクロールの脱メチル化
栗原記夫, ーノ顧礼司, 高尾佐知子
コラーゲナーゼ法によりラット肝細胞を分離し、これを用いて重水素化メトキシクロールを脱メチルさせた。メトキシクロールの両方のメチル基を重水素化した$d_3$体と片方のみメチル化した$d_4$体を調製して用いた。後者は (R)-, (S)-, (RS)-の3種を反応に用い、代謝物はモノ脱メチル体と少量のジ脱メチル体であった。反応速度およびその同位体効果を測定し、この脱メチル化反応は (1) ある程度のエナンチオ場選択性を有することと、(2) 有意に1よりも大きい重水素同位体効果を示すことを見いだした。