BIOTRANSFORMATION OF NITROSO AROMATIC COMPOUNDS AND 2-OXO ACIDS TO N-HYDROXY-N-ARYLACYLAMIDES BY THIAMINE-DEPENDENT ENZYMES IN RAT LIVER

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ABSTRACT:
The formation of N-hydroxy-N-arylacylamides from nitroso aromatic compounds and 2-oxo acids was investigated using rat liver subcellular fractions. Activities were found in both mitochondria and cytosol, except for activities for phenylpyruvate and glyoxylate; the former did not produce N-hydroxy-N-phenylphenylacetamide and the latter nonenzymatically produced N-hydroxy-N-phenylformamide with nitrosobenzene (NOB). The cytosolic activity of N-hydroxy-N-phenylglycolamide formation was indicated to be due to transketolase, which utilized hydroxypropyruvate as a glycolic aldehyde donor to NOB. With mitochondria, 2-oxo acids (including hydroxypropyruvate) served as substrates for the biotransformation of NOB to the corresponding N-hydroxy-N-arylacylamides. The substrate preference was 2-oxoaldehyde > pyruvate > 2-oxoisovalerate  2-oxoisocaproate > 2-oxovalerate > 2-oxo-3-methylvalerate, judging from V_{max}/half-saturating concentration for mitochondria values. The half-saturating concentrations for NOB were nearly constant. The mitochondrial activity was due to pyruvate dehydrogenase complex and branched-chain 2-oxo acid dehydrogenase complex (BCDHC). By using partially purified BCDHC, pyruvate and 2-oxoaldehyde were found to be common substrates for both of the enzymes, and 2-oxoisovalerate was shown to be the most effective substrate for BCDHC. Analysis by the Taft equation indicated that the polar effects, rather than the steric effects, of the alkyl groups of 2-oxo acids are important for BCDHC-catalyzed formation of N-hydroxy-N-phenylglycolamides. A positive Hammett constant obtained for the formation of N-hydroxy-N-arylisobutyrilamides indicates that an electron- withdrawing substituent makes the nitroso compounds susceptible to BCDHC-catalyzed biotransformation.

In the carcinogenicity of N-substituted aromatic compounds, proximate carcinogenic N-hydroxy-N-arylacetamides are thought to play important roles, because it has been shown that they can be activated to the electrophilic ultimate carcinogens capable of covalent binding to DNA (Goldstein and Faletto, 1993; Hanna, 1994; Michejda and Kroeger-Koepke, 1994). The formation of these proximate carcinogens from nitroso aromatic compounds has been reported to be mediated by thiamine-dependent enzymes (Corbett and Corbett, 1995), i.e. cytosolic transketolase (Corbett et al., 1979; Corbett and Corbett, 1986), pyruvate decarboxylase (Corbett and Chipko, 1980), mitochondrial PDHC1 (Yoshioka et al., 1989, 1996), and 2-oxoglutarate dehydrogenase complex (Corbett et al., 1982). In our previous studies, PDHC-catalyzed formation of N-hydroxy-N-arylacetamides was shown to proceed in isolated mammalian cells, including spermatogonia, and in perfused rat liver and heart (Yoshioka et al., 1989, 1996). Furthermore, the first component enzyme of PDHC, pyruvate decarboxylase, has been shown to be the enzyme responsible for the activity (Yoshioka and Uematsu, 1993).

In mitochondria, BCDHC, an enzyme complex analogous to PDHC, has been shown to participate in the metabolism of branched-chain 2-oxo acids derived from valine, leucine, and isoleucine (Harris et al., 1986; Yeaman et al., 1988; Yeaman, 1989). Because BCDHC is structurally and functionally similar to PDHC, it is of interest to investigate whether BCDHC catalyzes the formation of N-hydroxy-N-arylacylamides from nitroso aromatic compounds and branched-chain 2-oxo acids.

According to the proposed mechanism for the formation of N-hydroxy-N-arylacylamides (Corbett and Corbett, 1995; Yoshioka and Uematsu, 1993), N-hydroxy-N-arylglycolamides (products formed through a transketolase-mediated reaction of nitroso aromatic compounds with fructose-6-phosphate) (Corbett and Corbett, 1986) can be produced from nitroso aromatic compounds and hydroxypropyruvate. In this study, the abilities of rat liver subcellular fractions to catalyze the

1 Abbreviations used are: PDHC, pyruvate dehydrogenase complex; BCDHC, branched-chain 2-oxo acid dehydrogenase complex; MOPS, 3-morpholinopropanesulfonic acid; NOB, nitrosobenzene; S_{0.5}, half-saturating concentration for mitochondria; TPP, thiamine pyrophosphate; Me_{5}SO, dimethylsulfoxide; Me_{5}Si, tetramethylsilane; MeOH, methanol; EtOH, ethanol.

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Fig. 1. Biotransformation of nitroso aromatic compounds and 2-oxo acids to N-hydroxy-N-arylacylamides.

R is H, CH_{3}, CH_{2}OH, CH_{2}CH_{2}OH, CH_{2}CH_{2}CH_{2}OH, CH(CH_{3})_{2}, CH_{2}CH(CH_{3})_{2}, or CH(CH_{2})_{2}CH_{3}. X is H, m-Cl, p-Cl, p-CH_{3}, or p-ethoxy.

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formation of N-hydroxy-N-arylacylamides from nitroso aromatic compounds and several kinds of 2-oxo acids were investigated (fig. 1). The aim of this study was to evaluate the contribution of subcellular fractions to the formation of N-hydroxy-N-arylacylamide and to establish a structure-activity relationship for the biotransformation catalyzed by these thiamine-dependent enzymes.

**Experimental Procedures**

**Instrumentation.** 1H-NMR, MS, and IR spectra were recorded on JEOL JNM-GX270, Hitachi M-2000, and JASCO A-102 spectrometers, respectively. Measurement of UV spectra and spectrophotometric enzyme assays were carried out with a Shimadzu UV-200S spectrophotometer. HPLC was performed with a Shimadzu LC-6A liquid chromatograph equipped with a LiChrocart 250–4 cartridge column (LiChrosorb RP-8, 7 μm; Merck).

**Materials.** Sodium pyruvate was purchased from Merck (Darmstadt, Germany). Glyoxylic acid, TPP, lipoidamide dehydrogenase (from porcine heart), lithium hydroxypropionate, and sodium salts of 2-oxobutyrate, 2-oxoisovalerate, 2-oxoisocaproate, (p-2-oxo-3-ethyl)-2-oxobutyrate, 2-oxoisovalerate, 2-oxoisocaproate, (m-p-chlorophenyl)isobutyramide: m.p. 72–73°C (white plates from ethyl acetate/hexane); analysis calculated for C_{11}H_{15}NO_{2}: C, 68.37; H, 7.82; N, 11.30; found: C, 68.23; H, 7.93; N, 11.29; 1H-NMR (Me_{4}SO-d_{6}, Me_{3}Si): δ 1.08 (6H, d, J = 6.9 Hz), 2.29 (3H, s), 3.15 (1H, m), 7.16 (2H, d, J = 8.2 Hz), 7.46 (2H, d, J = 8.2 Hz), and 10.38 (1H, s); IR (Nujol) ν_{max}: 3170, 1620, 1460, and 1280 cm^{-1}; UV (EtOH) λ_{max}: 256 nm (ε = 9750); MS: m/z 193 (M^{+}), 177, 123, and 107 (base peak); N-hydroxy-N-(p-ethoxyphenyl)isobutyramide: m.p. 72–73°C (white plates from ethyl acetate/hexane); analysis calculated for C_{11}H_{15}NO_{2}: C, 64.56; H, 6.77; N, 6.27; found: C, 64.58; H, 7.78; N, 6.20; 1H-NMR (Me_{4}SO-d_{6}, Me_{3}Si): δ 1.07 (6H, d, J = 6.6 Hz), 1.32 (3H, t, J = 6.9 Hz), 3.16 (1H, m), 4.01 (2H, q, J = 6.9 Hz), 6.91 (2H, d, J = 9.2 Hz), 7.42 (2H, d, J = 9.2 Hz), and 10.36 (1H, s); IR (Nujol) ν_{max}: 3150, 1610, 1460, and 1250 cm^{-1}; UV (EtOH) λ_{max}: 257 nm (ε = 9680); MS: m/z 223 (M^{+}), 207, 153, 137 (base peak), and 108. All other chemicals used were of reagent grade.

**Subcellular Fractionation.** Male Wister rats (SLC Japan, Shizuoka, Japan), weighing between 120 and 130 g, were allowed free access to standard laboratory rat chow (25% protein by weight; Oriental Yeast Co.) and water. After the animals had been killed, the liver was removed, minced, and suspended in 9 volumes of ice-cold 0.25 M sucrose containing 3 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid-KOH (pH 7.4) and 0.1 mM EDTA disodium salt. The liver was homogenized in a Teflon glass homogenizer, with four vertical passes at low speed. The homogenates were combined and filtered through two layers of gauze. After centrifugation of the filtrate at 600 g for 10 min, the supernatant was centrifuged at 9000 g for 20 min, to sediment a mitochondrial pellet. The supernatant was then centrifuged at 105,000 g for 60 min, to yield a microsomal pellet and cytosol. The microsomal and mitochondrial fractions were suspended in the medium used for homogenization, and the resultant suspensions were stored at −80°C until use. The cytosol was concentrated by ultrafiltration (Amicon YM-10 filters; Amicon, Beverly, MA) and then dialyzed against 0.15 M KCl. The resultant dialyzed fraction was stored at −80°C until use. Protein was determined by a modification of the Lowry procedure (Markwell et al., 1981), with bovine serum albumin as the standard. The protein concentrations of microsomal and mitochondrial suspensions and dialyzed cytosol were 16.7 ± 0.1, 21.3 ± 0.2, and 7.80 ± 0.06 mg of protein/ml, respectively.

**Partial Purification of BCDHC from Rat Liver Mitochondria.** According to the procedure developed to purify BCDHC from rat liver (Shimomura et al., 1987), partial purification of BCDHC (free of PDHC), from liver mitochondrial of male Wister rats (200–210 g; SLC Japan), was performed by precipitation with polyethylene glycol, followed by phenyl-Sepharose separation. The column fractions with BCDHC activity were combined and concentrated by ultrafiltration (Centriplus-10 filters; Amicon). The concentrated solution was used for the experiments.

**Assay of BCDHC Activity.** BCDHC-catalyzed NADH formation activity was assayed spectrophotometrically according to a reported procedure (Paxton et al., 1986). Activity was assayed at 37°C, in a total volume of 1 ml, at pH 6.9 with 100 mM MOPS-KOH, 2 mM MgCl_{2}, 0.4 mM TPP, 0.4 mM CoA, 3 mM NAD^{+}, 0.1% (w/v) Triton X-100, 2 mM dihydrothreitol, 5 units of lipoidamide dehydrogenase, and 10 mM 2-oxoisovalerate.

**Assay of N-Hydroxy-N-arylacylamide Formation Activity.** The activity of the formation of N-hydroxy-N-arylacylamides was assayed according to the previously reported procedure (Yoshioka and Uematsu, 1993), with a slight modification. The assay medium (final volume, 1 ml), consisting of 100 mM MOPS-KOH (pH 6.9), 0.2 mM TPP, 2 mM MgCl_{2}, 0.2–2 mM nitroso aromatic compound [added as 25 μl of a bis(2-methoxyethyl)ether solution], and 10 mM pyruvate (40 mM for the other 2-oxo acids), was placed in a 2-ml screw-cap vial equipped with a Teflon-faced seal to prevent nitroso compound volatilization (Corbett and Corbett, 1986). After preincubation for 3 min, incubation was started by the addition of both substrates (2-oxo acid followed by nitroso aromatic compound within 15 sec) and
of mitochondria, seem to be the result of contamination by mitochondria. Because nondialyzed cystosol showed activity in the formation of N-hydroxy-N-phenylglycolamide from NOB by endogenous ketones (data not shown), dialyzed cystosol was used in this study. The dialed cystosolic activity in the formation of the glycolamide derivative is thought to be due to transketolase, as was reported by Corbett and colleagues (Corbett et al., 1979; Corbett and Corbett, 1986). The activity observed with fructose-6-phosphate (10 mM), one of the well-known glycolic aldehyde donors of transketolase, was higher (∼9-fold) than that measured with hydroxypyruvate (data not shown).

**Requirements for the Activity of Mitochondria.** Because the mitochondrial activity in the formation of N-hydroxy-N-phenylisobutyramide and its isobutyramide derivative were enhanced by both Mg²⁺ and TPP. These metabolites were not detected without the 2-oxo acids or NOB, and heat-treated mitochondria did not show any activity. Maximal activities were observed with 0.2 mM TPP, 2 mM MgCl₂, and 10 mM pyruvate or 40 mM 2-oxoisovalerate (data not shown).

**Mitochondrion-Catalyzed Formation of N-Hydroxy-N-phenylacrylamides.** To investigate the specificity for 2-oxo acids to serve as substrates in the mitochondrion-catalyzed formation of the corresponding N-hydroxy-N-phenylacrylamides, the reactivity of NOB with eight kinds of 2-oxo acids, including pyruvate and 2-oxoisovalerate, was examined. Of the tested 2-oxo acids, phenylpyruvate did not serve as a substrate for the formation of N-hydroxy-N-phenylacrylamide, although complicated nonenzymatic reaction products were obtained (data not shown). Kinetic constants for the formation of N-hydroxy-N-phenylacrylamides are summarized in table 4. S₀.₅ was used instead of Kₙ for reasons because two enzymes, i.e., PDHC and BCDHC, participate in the mitochondrion-catalyzed biotransformation, as described above. Therefore, glyoxyl acid has been shown to react nonenzymatically with NOB to yield N-hydroxy-N-phenylacrylamide (Corbett and Corbett, 1980), the second order rate constant is listed. With the other 2-oxo acids listed, the order of Vₚmax/S₀.₅ values (parameters indicating the catalytic efficiency of the formation of N-hydroxy-N-phenylacrylamides) was as follows: 2-oxobutyrate > pyruvate > 2-oxoisovalerate > 2-oxoisocaproate > 2-oxovalerate > 2-oxo-3-methylvalerate.

Because the 2-oxoglutarate dehydrogenase complex, located in mitochondria, has been shown to catalyze the formation of a succinimide acid derivative from a nitroso aromatic compound and 2-oxoglutarate (Corbett et al., 1982), the participation of the enzyme complex in the mitochondrion-catalyzed formation of N-hydroxy-N-phenylacrylamide and its isobutyramide derivative was investigated.

**TABLE 1**

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Time (min)</th>
<th>Elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. A, MeOH/H₂O, 3:7 (v/v);</td>
<td>0-12</td>
<td>Linear gradient to 24% B</td>
</tr>
<tr>
<td>B, MeOH/H₂O, 4:1 (v/v)</td>
<td>12-15</td>
<td>Linear gradient to 100% B</td>
</tr>
<tr>
<td>15-19</td>
<td>100% B</td>
<td></td>
</tr>
<tr>
<td>19-22</td>
<td>Linear gradient to 100% A</td>
<td></td>
</tr>
<tr>
<td>II. A, MeOH/H₂O, 1:4 (v/v);</td>
<td>0-9</td>
<td>100% A</td>
</tr>
<tr>
<td>B, MeOH/H₂O, 4:1 (v/v)</td>
<td>9-12</td>
<td>Linear gradient to 100% B</td>
</tr>
<tr>
<td>12-17</td>
<td>100% B</td>
<td></td>
</tr>
<tr>
<td>17-19</td>
<td>Linear gradient to 100% A</td>
<td></td>
</tr>
<tr>
<td>III. A, MeOH/H₂O, 4:5 (v/v)</td>
<td>0-13</td>
<td>100% A</td>
</tr>
<tr>
<td>B, MeOH/H₂O, 4:1 (v/v)</td>
<td>13-15</td>
<td>Linear gradient to 100% B</td>
</tr>
<tr>
<td>15-20</td>
<td>100% B</td>
<td></td>
</tr>
<tr>
<td>20-22</td>
<td>Linear gradient to 100% A</td>
<td></td>
</tr>
</tbody>
</table>

Solvant system I was used for N-hydroxy-N-phenylacetamide (R₂ = 6.1 min), propionamide (R₂ = 8.8 min), and isobutyramide (R₂ = 12.1 min) derivatives. Solvent system II was used for N-hydroxy-N-phenylglycolamide (R₂ = 4.9 min). Solvent system III was used for N-hydroxy-N-phenylisovaleramide (R₂ = 9.8 min).

**TABLE 2**

<table>
<thead>
<tr>
<th>2-Oxo Acid</th>
<th>R</th>
<th>Mitochondria</th>
<th>Microsomes</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxypyruvate</td>
<td>CH₃OH</td>
<td>35.2 ± 2.4</td>
<td>0.9 ± 0.2</td>
<td>32.1 ± 1.8</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>CH₃</td>
<td>28.5 ± 0.7</td>
<td>0.6 ± 0.1</td>
<td>21.2 ± 0.2</td>
</tr>
<tr>
<td>2-Oxoisovalerate</td>
<td>CH₃(CH₂)₂</td>
<td>27.3 ± 1.2</td>
<td>0.6 ± 0.1</td>
<td>21.1 ± 0.2</td>
</tr>
<tr>
<td>2-Oxoisocaproate</td>
<td>CH₃CH₂(CH₂)₂</td>
<td>16.7 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

The activities are expressed as the total activities of each fraction based on the initial velocities of the formation of N-hydroxy-N-phenylacrylamides, which were determined with 2 mM NOB, as described in Experimental Procedures.

* Cytosol refers to dialyzed cystosol.

**Results**

**N-Hydroxy-N-phenylacrylamide Formation by Rat Liver Subcellular Fractions.** The formation of N-hydroxy-N-phenylacrylamides from NOB and four kinds of 2-oxo acids (pyruvate, hydroxypyruvate, 2-oxoisovalerate, and 2-oxoisocaproate) was investigated in the presence of rat liver subcellular fractions, i.e., microsomes, mitochondria, and dialyzed cystosol. The activities of each fraction are summarized in table 2. With mitochondria, the corresponding N-hydroxy-N-phenylacrylamides were formed, although the activities were affected by 2-oxo acids. The microsomal activities, being roughly parallel to those of mitochondria, seem to be the result of contamination by mitochondria. Because nondialyzed cystosol showed activity in the formation of N-hydroxy-N-phenylglycolamide from NOB by endogenous ketones (data not shown), dialyzed cystosol was used in this study. The dialyzed cystosolic activity in the formation of the glycolamide derivative is thought to be due to transketolase, as was reported by Corbett and colleagues (Corbett et al., 1979; Corbett and Corbett, 1986). The activity observed with fructose-6-phosphate (10 mM), one of the well-known glycolic aldehyde donors of transketolase, was higher (∼9-fold) than that measured with hydroxypyruvate (data not shown).
TABLE 3

Requirements for N-hydroxy- N-phenylacylamide formation by rat liver mitochondria

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Relative Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R = CH&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>−2-Oxo acid</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>−Mg&lt;sup&gt;2+&lt;/sup&gt; (2 mM)</td>
<td>86</td>
</tr>
<tr>
<td>−TPP (0.2 mM)</td>
<td>72</td>
</tr>
<tr>
<td>−NOB (2 mM)</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>−Mitochondria</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heated mitochondria</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activities in the formation of N-hydroxy- N-phenylacylamide (R = CH<sub>4</sub>) and N-hydroxy- N-phenylisobutyramide (R = CH(CH<sub>3</sub>)<sub>2</sub>) are expressed relative to that of the complete mixture, arbitrarily taken as 100%. Heated mitochondria were prepared by heating for 20 sec in boiling water saturated with ammonium sulfate.

<sup>b</sup> ND, not determined.

As shown in table 5, the activities of mitochondria in the formation of acetamide and its isobutyramide derivative were not affected by the addition of 2-oxoglutarate.

Activities of Partially Purified BCDHC in the Formation of N-Hydroxy- N-phenylacylamides. BCDHC has been shown to catalyze the oxidative decarboxylation of pyruvate and 2-oxobutyrate, as well as branched-chain 2-oxo acids (Paxton et al., 1986; Paxton and Harris, 1982). Porcine heart PDHC has been reported to catalyze the biotransformation of NOB to N-hydroxy- N-phenylacetamide and its propionamide derivative in the presence of pyruvate and 2-oxobutyrate, respectively (Yoshioka and Uematsu, 1993). From these findings, the mitochondrion-catalyzed formation of N-hydroxy- N-phenylacetamide and its propionamide derivative is thought to be mediated by both PDHC and BCDHC. Therefore, substrate specificities for 2-oxo acids in the formation of N-hydroxy- N-phenylacylamides were investigated with a rat liver BCDHC preparation free of PDHC.

As shown in fig. 2, among the 2-oxo acids tested, 2-oxosovalerate (fig. 2, point a) (yielding N-hydroxy- N-phenylisobutyramide) was the most effective substrate for partially purified BCDHC-catalyzed biotransformation. The activities in the formation of isobutyramide (fig. 2, point a), 2-methylbutyramide (fig. 2, point c), isovaleramide (fig. 2, point d), and butyramide (fig. 2, point e) derivatives correlated well (£r = 0.996, N = 4) with those observed with mitochondria. These results indicate that the formation of the aforementioned four metabolites is catalyzed by BCDHC but not by PDHC. The activities of mitochondria in the formation of N-hydroxy- N-phenylpropionamide (fig. 2, point b’) and its acetamide derivative (fig. 2, point f’) were higher than those (fig. 2, points b and f) calculated from the correlation equation shown in the legend to fig. 2. The enhanced activities are thought to be the result of PDHC in mitochondria.

As shown in fig. 3, a correlation for log(BCDHC activity) vs. the £s<sup>a</sup> (polar effect) and £s<sup>b</sup> (steric effect) parameters of the R groups of 2-oxo acids was observed using the Taft equation (Shorter, 1972). The correlation equation (£r = 0.912, N = 6) is as follows:

\[
\log(\text{BCDHC activity}) = 0.27\,E_s - 3.10\,\sigma^* + 1.42
\]

(0.66) (1.27)

From the standard partial regression coefficients shown in parentheses in the above equation, £s<sup>a</sup> was found to be more important than £s<sup>b</sup>. The negative value (−3.10) of £s indicated the activity of BCDHC-catalyzed formation of N-hydroxy- N-phenylacylamides increases with increases in the electron-donating ability of the R group of 2-oxo acids.

As reported by Shimomura et al. (1987), the addition of lipoamide dehydrogenase was necessary for full expression of the partially purified BCDHC-catalyzed NADH formation activity. The activity in the formation of N-hydroxy- N-phenylisobutyramide, however, was not affected by the addition of lipoamide dehydrogenase (data not shown).

Effects of NOB Substituents on Activity. Several nitroso aromatic compounds were studied by using mitochondria to elucidate the effect of ring substituents on BCDHC-catalyzed formation of N-hydroxy- N-arylisobutyramides. Because the formation of N-hydroxy- N-arylisobutyramides was shown to be mediated by BCDHC but not by PDHC (fig. 2), the half-saturating concentrations for nitroso aromatic compounds tested in mitochondria are shown as $K_M$ values in table 6. Although the $K_M$ values were nearly constant, $V_{max}$ values were affected by both the ring substituents and their position. As shown in fig. 4, a correlation with the positive £s value was observed in the Hammett plot (Hammett, 1970) of log($V_{max}/K_M$) vs. £s. The equation (£r = 0.989, N = 5) is as follows:

\[
\log V_{max}/K_M = (0.735 \pm 0.067)\sigma^* + (0.539 \pm 0.028)
\]

This result indicates that nitroso aromatic compounds serve as electrophiles in their biotransformation to N-hydroxy- N-arylacrylamides.

### Table 4

<table>
<thead>
<tr>
<th>2-Oxo Acid</th>
<th>R</th>
<th>$S_{65}$</th>
<th>$V_{max}$</th>
<th>$V_{max}/S_{65}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyoxylate</td>
<td>H</td>
<td>0.332 ± 0.038</td>
<td>1.51 ± 0.07</td>
<td>0.132 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>CH</td>
<td>0.394 ± 0.014</td>
<td>2.12 ± 0.03</td>
<td>5.38</td>
</tr>
<tr>
<td>2-Oxobutyrate</td>
<td>CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.287 ± 0.024</td>
<td>0.704 ± 0.028</td>
<td>2.45</td>
</tr>
<tr>
<td>2-Oxovalerate</td>
<td>CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.351 ± 0.011</td>
<td>1.43 ± 0.06</td>
<td>4.07</td>
</tr>
<tr>
<td>2-Oxosuccinate</td>
<td>CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.310 ± 0.060</td>
<td>0.775 ± 0.050</td>
<td>2.50</td>
</tr>
<tr>
<td>2-Ox-3-methylvalerate</td>
<td>CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.380 ± 0.011</td>
<td>0.864 ± 0.070</td>
<td>2.27</td>
</tr>
</tbody>
</table>

<sup>a</sup> Second order rate constant (M<sup>−1</sup> sec<sup>−1</sup>) for the nonenzymatic formation of N-hydroxypyridineformamidine at 37°C and pH 6.9. The reported value (Corbett and Corbett, 1980) measured at 30°C and pH 6.0 was 0.119 ± 0.010 M<sup>−1</sup> sec<sup>−1</sup>.
cytosol-catalyzed formation of N-droxy-N-phenylacrylamide served as a substrate for both the mitochondrion- and lamriderivative was formed also with fructose-6-phosphate, as reported. Cytosolic activity is thought to be due to transketolase, because the glyco-

6

sult obtained with porcine heart PDHC (Yoshioka and Uematsu, 1993). As shown in fig. 3, it is thought that the activity of BCDHC-catalyzed formation of N-hydroxy-N-phenylacrylamidemands affected by the polar effects, rather than the steric effects, of the R

Discussion
Formation of N-hydroxy-N-arylacylamides from nitroso aromatic compounds and 2-oxo acids has been observed in rat liver mitochondria and cytosol. Phenylpyruvate did not serve as a substrate for the corresponding N-hydroxy-N-phenylphenylacetamide. Glyoxylate reacted non-

enzymatically with NOB to yield N-hydroxy-N-phenylformamide. Hydroxy
ypyruvate served as a substrate for both the mitochondrion- and cytosol-catalyzed formation of N-hydroxy-N-phenylglycynamide. The
cytosolic activity is thought to be due to transketolase, because the glyco-
lamide derivative was formed also with fructose-6-phosphate, as reported by Corbett and colleagues (Corbett et al., 1979; Corbett and Corbett, 1986), and fructose-6-phosphate and hydroxypyruvate are known to be donors of glycolic aldehyde (Kochetov, 1982). The activity observed with mitochondria in the formation of the glycynamide derivative is thought to be due to PDHC, because hydroxypyruvate has been shown to be a substrate in PDHC-catalyzed reduction of 2,6-dichlorophenoldiolophenol (Khailova et al., 1989).

As shown in fig. 2, pyruvate and 2-oxobutyrate were substrates for PDHC-catalyzed biotransformation of NOB to the corresponding N-hydroxy-N-phenylacrylamides. This result does not contradict the result obtained with porcine heart PDHC (Yoshioka and Uematsu, 1993). These two 2-oxo acids were also shown to be common sub-

strates for BCDHC. From the activities shown in fig. 2, the activities for PDHC-catalyzed formation of N-hydroxy-N-phenylacrylamide and its propionamide derivative were calculated to be 1.03 and 0.92 nmol/min/mg, respectively. The ratio (0.89) of the activities is comparable to that (0.82) reported with porcine heart PDHC (Yoshioka and Uematsu, 1993). It appears that PDHC plays a greater role in the mitochondrial-catalyzed formation of N-hydroxy-N-arylacylamides than does BCDHC, because the calculated activity of PDHC (1.03 nmol/min/mg) is 75% of the activity of mitochondria (PDHC + BCDHC) (1.38 nmol/min/mg).

As shown in table 5, the addition of 2-oxoglutarate did not affect the mitochondrial activities in the formation of N-hydroxy-N-phenylacetamide and its isobutyramide derivative. Pig heart 2-oxoglutarate dehydrogenase complex has been reported to be very specific for the oxidation of 2-oxoglutarate (Koike et al., 1974). These findings suggest that the enzyme complex does not participate in the formation of N-hydroxy-N-arylacylamide and its isobutyramide derivative.

Compared with PDHC, BCDHC was shown to have a broad substrate specificity for 2-oxo acids. BCDHC participates in the biotransformation of NOB with pyruvate, 2-oxobutyrate, 2-oxovalerate, as well as bulky branched-chain 2-oxo acids (fig. 3). This result obtained with BCDHC seems to be different from that for PDHC, whose active center structure has been indicated to have steric limitations with respect to the β-substituent of pyruvate analogs (Khailova et al., 1989). As shown in fig. 3, it is thought that the activity of BCDHC-catalyzed formation of N-hydroxy-N-phenylacrylamides is affected by the polar effects, rather than the steric effects, of the R groups of 2-oxo acids. This result suggests that the active center structure of BCDHC is larger than that of PDHC, i.e., sufficiently large to be occupied by bulky branched-chain 2-oxo acids.

Because BCDHC-catalyzed formation of NADH has been reported to proceed through a ping-pong mechanism (Boyer and Odessey,
Corbett MD and Chipko BR (1980) Reaction of nitroso aromatics with glyoxylic acid: a new path
Corbett MD and Chipko BR (1979) Quantitative determination of
Corbett MD and Chipko BR (1980) Comparative aspects of hydroxamic acid production by

Because BCDHC activity has been reported to be found in rat heart, kidney, liver, diaphragm, brain, and quadriceps muscle (Wagenmakers et al., 1984), the formation of \(N\)-hydroxy-\(N\)-arylcyclamides could proceed in every tissue in mammals. Because thiamine-dependent enzymes, i.e. BCDHC, PDHC, and transketolase, play central roles particularly in the catabolism and anabolism of cells, the biotransformation of nitroso aromatic compounds catalyzed by these enzymes may exert toxic effects on all cells by disturbing the energy and material metabolism of the cells. Although additional work is needed, it is possible that the biotransformation of carcinogenic nitroso aromatic compounds into the corresponding proximate carcinogenic \(N\)-hydroxy-\(N\)-arylcyclamides by these thiamine-dependent enzymes is responsible for carcinogenicity and/or necrosis in tissues.

References


