The human liver $\alpha\alpha$ alcohol dehydrogenase exhibits a different substrate specificity and stereospecificity for secondary alcohols than the human $\beta\beta$, and $\gamma\gamma$ or horse liver alcohol dehydrogenases. All of the enzymes efficiently oxidize primary alcohols, but $\alpha\alpha$ oxidizes secondary alcohols far more efficiently than human $\beta\beta$, and $\gamma\gamma$ or horse liver alcohol dehydrogenase. Specifically, $\alpha\alpha$ oxidizes four- and five-carbon secondary alcohols with efficiencies up to 3 orders of magnitude greater than those of the three other isoenzymes. Whereas the human $\beta\beta$, and $\gamma\gamma$, and horse isoenzymes show a distinct preference toward (S)-(+) 3-methyl-2-butanol, the $\alpha\alpha$ isoenzyme prefers (R)-(−)-3-methyl-2-butanol. Computer-simulated graphics demonstrate that the horse subunit accommodates (S)-(+) 3-methyl-2-butanol within the active site much better than the opposite stereoisomer, primarily due to steric hindrance caused by Phe-93. Human $\alpha$ may accommodate (R)-(−)-3-methyl-2-butanol better than (S)-(+) 3-methyl-2-butanol because of close contacts between the latter and Thr-48. These observations suggest that substitutions at positions 93 and 48 in the active site of human liver alcohol dehydrogenase isoenzymes may determine their substrate specificity for secondary alcohols.

Horse liver alcohol dehydrogenase (alcohol: NAD$^+$ oxidoreductase, EC 1.1.1.1) exhibits broad substrate specificity and oxidizes secondary as well as primary alcohols (1, 2). In addition, this enzyme exhibits stereospecificity for oxidation of secondary alcohols and their formation from ketones. The enzyme prefers substituted cyclohexanols in the $S$ configuration over those in the inverted configuration (3–5).

The structure of the substrate-binding site of horse liver alcohol dehydrogenase has been determined by x-ray crystallography of several complexes of the enzyme with coenzyme and substrate (e.g. NAD$^+$ and bromobenzyl alcohol (6)), or inhibitors (e.g. NADH and dimethyl sulfoxide (7); NAD$^+$ and pyrazole (8)). These structural studies indicate that the substrate-binding site is lined with hydrophobic residues including Phe-93. This bulky amino acid is believed to play an important role in substrate specificity for both primary (6) and secondary (3) alcohols, presumably by sterically blocking the binding of alcohols. Other amino acids that line the alcohol-binding cleft and that may affect substrate specificity include Ser-48, Leu-57, Phe-110, Leu-116, Leu-141, and Ile-318 (7).

Class I human liver isoenzymes of alcohol dehydrogenase, those with $\alpha$, $\beta$, or $\gamma$ subunits, are at least 87% homologous with the horse liver enzyme (9–13). Thirty-three amino acids comprise the alcohol and NAD(H)-binding domains of horse alcohol dehydrogenase (7), and these amino acids are highly conserved in the human class I isoenzymes. The $\alpha$, $\beta$, and $\gamma$ subunits of human Class I alcohol dehydrogenase differ from horse alcohol dehydrogenase at 9, 7, and 5, of these 33 positions, respectively.

Eklund and co-workers (14) predicted that human $\alpha\alpha$ may oxidize secondary alcohols with greater efficiency than human $\beta\beta$, or $\gamma\gamma$, or horse alcohol dehydrogenases because $\alpha\alpha$ contains an alanine at position 93, whereas the other alcohol dehydrogenases contain phenylalanine. The authors also predicted that the Thr-48 substitution in $\alpha\alpha$ for serine could affect substrate specificity. Except for the symmetrical cyclohexanol, specificities of human isoenzymes have not been determined toward secondary alcohols. We have therefore examined the substrate specificities of human liver $\alpha\alpha$, $\beta\beta$, $\gamma\gamma$, and horse liver alcohol dehydrogenase for primary alcohols and isoenzymes of secondary alcohols.

### EXPERIMENTAL PROCEDURES

**Reagents**—NAD$^+$ (Grade III) and horse alcohol dehydrogenase were purchased from Boehringer Mannheim. (R)-(−) and (S)-(+) 2-pentanol and (R)-(−)-3-methyl-2-butanol were purchased from Chemalog. (S)-(+) 3-Methyl-2-butanol was purchased from Sigma. (R)-(−) and (S)-(+) 2-butanol were purchased from Aldrich. Purity of the enantiomers was confirmed by polarimetry. All other reagents were purchased from Sigma, and all reagents were of the highest grade commercially available.

**Enzyme Preparation**—Purification procedures for human liver alcohol dehydrogenase isoenzymes $\alpha\alpha$, $\beta\beta$, and $\gamma\gamma$ have been described (15). All human isoenzymes were judged homogeneous by starch and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16) and agarose isoelectric focusing (17). Each isoenzyme was prepared for kinetic studies by passage over a Bio-Gel P-6 DG (Bio-Rad) column equilibrated with 10 mM sodium phosphate, pH 8.5. Horse alcohol dehydrogenase was prepared by gel filtration on Bio-Gel P-6 DG in 10 mM sodium phosphate, pH 8.5, after dialysis of the ammonium sulfate suspension. Polyacrylamide gel electrophoresis demonstrated that the horse preparation contained smaller molecular weight species, presumably from protein degradation, but amounting to less than 5% of the sample. Protein concentrations were determined by Lowry and co-workers (18) using bovine serum albumin as a standard. Enzyme activity was assayed in 0.1 M glycine, pH 10.0, with 35 mM ethanol and 2.4 mM NAD$^+$ as substrates. The average specific activity obtained for each enzyme was as follows: human $\alpha\alpha$, 1.7; $\beta\beta$, 0.24; $\gamma\gamma$, 4.7; and horse, 3.0 units/mg.

**Kinetics**—Enzyme kinetics with various primary and secondary alcohols were performed with a Gilford Instrument Laboratories, Inc. Response spectrophotometer by monitoring the production of NADH at 340 nm and utilizing an extinction coefficient of 6.22 mm$^{-1}$ cm$^{-1}$. Enzyme (0.5–13 μg/ml) and 2.4 mM NAD$^+$ in 0.1 M sodium phosphate.
TABLE I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}/K_m$ (units/mg/min)</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}/K_m$ (units/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>6100</td>
<td>222</td>
<td>330</td>
<td>320</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>32</td>
<td>22</td>
<td>40</td>
<td>65</td>
</tr>
<tr>
<td>1-Pentanol</td>
<td>14</td>
<td>13</td>
<td>20</td>
<td>41</td>
</tr>
<tr>
<td>Isobutyl alcohol</td>
<td>290</td>
<td>61</td>
<td>61</td>
<td>160</td>
</tr>
<tr>
<td>Isopentyl alcohol</td>
<td>210</td>
<td>14</td>
<td>160</td>
<td>530</td>
</tr>
</tbody>
</table>

* From Burnell and co-workers.}

**RESULTS**

**Specificity of Isoenzymes toward Primary Alcohols**—The Michaelis kinetic constant ($K_m$) and catalytic efficiency ($V_{max}/K_m$) of human liver $\alpha\alpha$, $\beta\beta$, $\gamma\gamma$, and horse liver alcohol dehydrogenase were determined toward increasing lengths of primary alcohols having straight and iso-chains (Tables I and II). For each enzyme, the $V_{max}$ values did not change appreciably among the straight chain substrates (data can be calculated from Tables I and II). Ethanol was a very poor substrate for $\alpha\alpha$, with a $K_m$ of 6.1 mM; a value of 4.2 mM was reported previously (21). As chain length increased, the $K_m$ of $\alpha\alpha$ for primary straight chain alcohols decreased dramatically, and the substrate efficiency increased. The decrease in $K_m$ toward straight chain alcohols (Table I) probably reflects a decrease in the substrate dissociation constant. The increasing efficiency with increasing chain length observed with $\alpha\alpha$ was also seen with the other enzymes studied (Tables I and II). Of all the isoenzymes studied, human $\gamma\gamma$ oxidized long chain primary alcohols most efficiently.

Human $\alpha\alpha$ exhibited $K_m$ values toward isobutyl alcohol and isopentyl alcohol that were 9 and 15 times greater than the straight chain alcohols of the same carbon number, and the enzyme oxidized isopentyl alcohol more efficiently than isobutyl alcohol. The $V_{max}$ of $\alpha\alpha$ toward isobutyl alcohol was reduced to about one-third of the value observed toward the other primary alcohols. The pattern of decreasing $K_m$ with increasing chain length was seen with the two iso-chain alcohols for $\beta\beta$, but not with human $\gamma\gamma$ or horse alcohol dehydrogenase. In these two cases, the $K_m$ increased with increasing chain length, producing a decrease in efficiency.

**Specificity of Isoenzymes toward Secondary Alcohols**—To evaluate the substrate specificity of human alcohol dehydrogenase isoenzymes toward secondary alcohols, the $K_m$ values and catalytic efficiencies toward two straight chain alcohols, one iso-chain secondary alcohol, and cyclohexanol were examined (Tables III and IV). The relative efficiencies toward $R$ versus $S$ configurations of secondary alcohols of each isoenzyme were also compared (Table V).

Most striking are the relatively low $K_m$ and high efficiency.

**A plot of log($V_{max}/K_m$) versus log $P$ value of each alcohol (Fig. 1) indicates that the efficiencies of both human $\gamma\gamma\gamma$ and $\alpha\alpha\alpha$ as well as horse alcohol dehydrogenase are linearly related to hydrophobicity of the alcohol (22). The $\alpha\alpha\alpha$ enzyme exhibited a slope greater than unity through a chain length of 4. This suggests that the efficiency of $\alpha\alpha\alpha$ is related not only to hydrophobicity but also to some additional amplifying effect (22, 23). This amplifying effect was not observed with human $\gamma\gamma\gamma$ and horse, where the slope was near unity (Fig. 1). The $V_{max}/K_m$ of human $\beta\beta\beta$ was relatively constant with respect to the substrate, a result reported by Burnell and co-workers.**

**TABLE II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{max}/K_m \times 10^4$ (units/mg/µM)</th>
<th>$\alpha\alpha\alpha$</th>
<th>$\beta\beta\beta$</th>
<th>$\gamma\gamma\gamma$</th>
<th>Horse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>0.67</td>
<td>42</td>
<td>37</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>1-Butanol</td>
<td>160</td>
<td>59</td>
<td>460</td>
<td>200</td>
<td>310</td>
</tr>
<tr>
<td>1-Pentanol</td>
<td>270</td>
<td>94</td>
<td>850</td>
<td>310</td>
<td></td>
</tr>
<tr>
<td>Isobutyl alcohol</td>
<td>5.7</td>
<td>17</td>
<td>190</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Isopentyl alcohol</td>
<td>20</td>
<td>57</td>
<td>47</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Residue 48</td>
<td>Thr</td>
<td>Thr</td>
<td>Ser</td>
<td>Ser</td>
<td></td>
</tr>
<tr>
<td>Residue 93</td>
<td>Ala</td>
<td>Phe</td>
<td>Phe</td>
<td>Phe</td>
<td></td>
</tr>
</tbody>
</table>

* From Burnell and co-workers.}

Fig. 1. Effect of substrate partition coefficient on isoenzyme catalytic efficiency. The partition coefficient of each substrate was obtained from Lee and co-workers (40). Ethanol, 1-butanol, and 1-pentanol were used to construct curves for human $\beta\beta\beta$, (C), $\gamma\gamma\gamma$, (A), and horse (O) enzymes. 1-Propanol and 1-heptanol substrates were additionally used to construct the curve for human $\alpha\alpha\alpha$ (D). Human $\gamma\gamma\gamma$ and horse alcohol dehydrogenase both display slopes near unity; human $\alpha\alpha\alpha$ displays a slope greater than unity; $\beta\beta\beta$ displays a slope near zero.
those alcohols in the (R)-(-) configuration. For example, the
isoenzymes, however, demonstrated a distinct preference to-
ward the alcohols at an approximately equal or greater efficiency than
alcohol dehydrogenase oxidized secondary alcohols 8 to almost
33 times less efficiently than the S-enantiomers. Human γ₁γ₁ oxidized the (R)-(−)-2-butanol more efficiently than the opposite stereoisomer (Table V), but it lagged far behind αα in efficiency with both stereoisomers (Table IV). With 3-
methyl-2-butanol, γ₁γ₁ oxidized the (R)-(−)-isomer more
than 5 times less efficiently than the (S)-(+) -isomer.

Of all the secondary alcohols tested, cyclohexanol was the
best substrate for αα. The Kₐ toward this alcohol was at least
4 times lower (Table III) and the efficiency 6 times higher (Table IV) than those values for the other secondary alcohols
examined with αα. The enzyme also oxidized cyclohexanol at least 2 times more efficiently than any of the primary alcohols
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other secondary alcohols studied with these enzymes. Only
ββ oxidized cyclohexanol poorly. These results are in agreement
with those of Merritt and Tomkins (1) and Wagner and
co-workers (24).

Whereas the Vₕ of primary alcohols was not generally
affected by increasing chain length, a variation in the Vₕ toward
cyclohexanol and 3-methyl-2-butanol was observed (data can be calculated from Tables III and IV). Relative to the Vₕ
max observed toward the other alcohols studied, human
ββ showed a 2-fold decrease in Vₕ max toward cyclohexanol,
whereas horse alcohol dehydrogenase exhibited an increase in Vₕ
max toward this substrate by 4 times. Human γ₁γ₁ showed a
decrease in Vₕ max toward both enantiomers of 3-methyl-2-
butanol, whereas αα demonstrated a reduced Vₕ max toward the
(S)-(+) -enantiomer of this substrate. Although the primary
alcohols exhibited decreasing Kₐ and increasing efficiency values with increasing chain length (Table I), no readily
apparent pattern in Kₐ was observed with increasing chain
length of secondary alcohols (Table III).

**DISCUSSION**

Examination of the substrate specificity of human and
horse alcohol dehydrogenases for primary and secondary
alcohols demonstrates that human αα alcohol dehydrogenase
oxidizes secondary alcohols far more efficiently than the
human liver ββ, γ₁γ₁, or horse liver alcohol dehydrogenases.
In sharp contrast to these isoenzymes, αα oxidizes the
(R)-(−)-configuration of these substrates, and in the case of
3-methyl-2-butanol, it prefers this configuration 17-fold over
the inverted configuration. Thus, the human αα isoenzyme is
unique in its ability to oxidize (R)-(−)-secondary alcohols.

Eklund and co-workers (6) used computer graphics to build
ethanol into the substrate-binding site of horse alcohol de-
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toward C₄ of the nicotinamide ring, the alcohol fits well within
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the inverted configuration. Thus, the human αα isoenzyme is
unique in its ability to oxidize (R)-(−)-secondary alcohols.
enantiomers of 3-methyl-2-butanol have the following configurations:

\[
\begin{align*}
\text{H}_2 & \quad \text{H} \\
\text{CH}_3 & \quad \text{CH}_2 \quad \text{H} \\
\text{CH}_3 & \quad \text{CH}_2 \quad \text{H} \\
\text{H} & \quad \text{H} \\
\text{or} & \quad (\text{CH}_3 \quad \text{CH}_2 \quad \text{H}) \\
\text{S} & \quad (\text{CH}_3 \quad \text{CH}_2 \quad \text{H}) \\
\text{S} & \quad (\text{CH}_3 \quad \text{CH}_2 \quad \text{H})
\end{align*}
\]

Extrapolating from Eklund’s conclusions, the S-enantiomer of a secondary alcohol should make a better substrate for horse alcohol dehydrogenase than the R-enantiomer. To illustrate this, computer graphics of the horse substrate-binding site were used. As shown in Fig. 2A, the bulky isopropyl side chain of (S)-(+-)-3-methyl-2-butanol is pointing away from the Phe-93 and toward Ser-48. This permits the substrate to fit into the binding pocket. Binding of the R-enantiomer into the pocket, however, would position the isopropyl group toward Phe-93 and produce very tight interactions. The kinetic results presented in Table V support this prediction.

Dickinson and Dalziel (27) observed that horse liver alcohol dehydrogenase oxidizes both (R)-(+-)- and (S)-(+-)-2-butanol but that the enzyme oxidizes the (S)-(+-)-enantiomer at a greater rate than the (R)-(+-)-alcohol. The kinetics of oxidation of secondary alcohol enantiomers reported here are in agreement with their results. These authors also demonstrated that yeast alcohol dehydrogenase is unable to oxidize secondary alcohols in the (R)-configuration. Jornvall and co-workers showed that although the yeast enzyme is only 25% homologous in amino acid sequence to horse alcohol dehydrogenase (28), the structure of the active site is similar (29). The amino acids in the yeast enzyme which correspond to positions Phe-93 and Ser-48 in horse alcohol dehydrogenase are tryptophan and threonine, respectively. Relative to the horse enzyme, these substitutions in the yeast enzyme decrease the size of the binding pocket further and may explain the inability of yeast alcohol dehydrogenase to oxidize R-secondary alcohols.

According to the lattice structure built for horse alcohol dehydrogenase by Irwin and Jones (30), it can be shown that the isopropyl group of (S)-(+-)-3-methyl-2-butanol fits into the substrate-binding pocket of the enzyme. Horjales and Brändén (31) expanded the lattice model and used computer-simulated graphics to predict the substrate specificity of substituted cyclohexanols with horse alcohol dehydrogenase. These authors predicted that whereas alcohols with both stereoisomers of substituted cyclohexanols and cyclohexanones. These results are consistent with those presented in Tables III and IV.

To simulate the $\alpha$ subunit by computer graphics and the effect of substitutions of Ala-93 for Phe-93, and Thr-48 for Ser-48, these and seven other amino acids in a 10 Å radius around the substrate of the horse structure were replaced. The (R)-(+-)-3-methyl-2-butanol was built into the simulated active site (Fig. 2B). Substitution of alanine for Phe-93 in human $\alpha\alpha$ alcohol dehydrogenase enlarges the binding pocket considerably, permitting ample room for the isopropyl group of the substrate. No close contacts between the $R$-alcohol and Ala-93 are observed. Substitution of threonine in $\alpha\alpha$ for Ser-48 in horse alcohol dehydrogenase narrows the binding pocket so that with (S)-(+-)-3-methyl-2-butanol, the isopropyl group would face toward Thr-48 and cause tight interactions. These observations suggest that both Phe-93 and Thr-48 contribute to the unique stereospecificities of human $\alpha\alpha$ alcohol dehydrogenase.

According to the above hypothesis, human isoenzymes $\pi$ and $\chi\chi$, which contain a tyrosine and threonine substitution at positions Phe-93 and Ser-48 in horse (32, 33), should not oxidize secondary alcohols very efficiently in either stereoisomeration. The studies of Vallee and Bazzone (34) indicate that these isoenzymes oxidize secondary alcohols much less efficiently than primary alcohols. For example, the $K_m$ toward cyclohexanol is 210 mM for $\pi\pi$, whereas activity of $\chi\chi$ toward cyclohexanol is not detectable even at 0.1 m. The stereospecificity of these isoenzymes toward secondary alcohols has not been reported.

Human $\alpha\alpha$ alcohol dehydrogenase is the predominant Class I isoenzyme found in the fetal liver throughout gestation. The predominance of this isoenzyme decreases during childhood development, as other isoenzymes are expressed in increasing amounts (35). With ethanol ingestion by the mother, the fetal concentration of ethanol very quickly approaches that of the mother (36). This indicates that the placenta, which contains high $K_m$ forms of alcohol dehydrogenase (37, 38), has little effect on metabolizing the drug prior to its transport into the fetus and surrounding amniotic fluid (39). As a result of the high $K_m$ and low efficiency of human $\alpha\alpha$ for ethanol, the ethanol concentration builds to a high level before it can be
Acknowledgments—Dr. David Gorenstein and James Metz (Center for Biomolecular NMR, Structure and Design, Purdue University) are gratefully acknowledged for their assistance with the Silicon liver alcohol dehydrogenase isoenzymes. Leslie Magnes and Kwabena Owusu-Dekyi for purifying the human liver alcohol dehydrogenase isoenzymes.

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