The Specificities and Configurations of Ternary Complexes of Yeast and Liver Alcohol Dehydrogenases

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1. Some aspects of the substrate specificities of liver and yeast alcohol dehydrogenases have been investigated with pentan-3-ol, heptan-4-ol, (+)-butan-2-ol, (±)-butan-2-ol, (±)-hexan-3-ol and (±)-octan-2-ol as potential substrates. The liver enzyme is active with all substrates tested, including both isomers of each optically active alcohol. In contrast, the yeast enzyme is completely inactive towards those secondary alcohols where both alkyl groups are larger than methyl and active with only the (+)-isomers of butan-2-ol and octan-2-ol. 2. The absence of stereospecificity of liver alcohol dehydrogenase towards optically active secondary alcohols and its broad specificity towards secondary alcohols in general are explained in terms of an alkyl-binding site that will react with a variety of alkyl groups and the ability of the enzyme to accommodate a fairly large unbound alkyl group in an active enzyme-NAD⁺-secondary alcohol ternary complex. The absolute optical specificity of the yeast enzyme towards *n*-alkylmethyl carbinols and its unreactivity towards pentan-3-ol, hexan-3-ol and heptan-4-ol are explained by its inability to accept alkyl groups larger than methyl in the unbound position in a viable ternary complex. 3. Comparison of the known configurations of the *n*-alkylmethyl carbinols and [1-³H]ethanol and [1-³H]geraniol, which have been used in stereospecificity studies with these enzymes by other workers, provides strong evidence for which alkyl group of the substrate is bound to the enzyme in the oxidation of *n*-alkylmethyl carbinols. The conclusions reached are, for butan-2-ol oxidation with the liver enzyme, confirmed by deductions from kinetic data obtained with (+)-butan-2-ol and a sample of butan-2-ol containing 66% of (-)-butan-2-ol. 4. Initial-rate parameters for the oxidations of (+)-butan-2-ol, 66% (-)-butan-2-ol and pentan-3-ol by NAD with liver alcohol dehydrogenase are presented. The data are completely consistent with a general mechanism of catalysis previously proposed for this enzyme.

On the basis of detailed kinetic studies with several alcohols, aldehydes and ketones as substrates (Dalziel & Dickinson, 1966*a,b*) a unified mechanism has been proposed for the reversible oxidation of primary, secondary and cyclic alcohols and the dismutation of aldehydes catalysed by liver alcohol dehydrogenase (alcohol-NAD⁺ oxidoreductase, EC 1.1.1.1). Initial-rate data for several primary alcohols (Dalziel, 1963*a*; Dalziel & Dickinson, 1966*a*) and for cyclohexanol (Dalziel & Dickinson, 1966*b*) are consistent with a limiting case of this general mechanism, the Theorell-Chance mechanism (Theorell & Chance, 1951), except with large substrate concentrations, whereas data for secondary alcohols are not. This is attributed primarily to the fact that the rate of intramolecular oxidation-reduction within the ternary complex of alcohol, NAD⁺ and enzyme is faster with

primary alcohols than with secondary alcohols. Substrate inhibition observed with primary alcohols, and substrate activation and inhibition exhibited by cyclohexanol in the presence of high and low coenzyme concentrations respectively, are accounted for by the formation of abortive ternary complexes of enzyme, alcohol and NADH, and of binary enzyme-alcohol complexes that combine with NAD⁺ more slowly than the free enzyme.

The broad specificity of liver alcohol dehydrogenase for the alkyl groups of primary and secondary alcohols, and its reactivity with cyclohexanol as substrate, suggested that it may not exhibit stereospecificity towards optically active secondary alcohols. Dickinson & Dalziel (1967) showed that both enantiomorphs of butan-2-ol are substrates and indeed, in low concentrations, do not differ greatly in activity. A more detailed account of

these experiments, and the results of similar experiments with $(\pm)$ -octan-2-ol, are given in the present paper. Since the precise significance of initial-rate parameters previously determined for $(\pm)$ -butan-2-ol is uncertain, initial-rate studies with the pure $(+)$ -isomer and partially resolved $(-)$ -isomer are also reported.

Unlike the liver enzyme, yeast alcohol dehydrogenase (EC 1.1.1.1) is stereospecific for the $(+)$ -isomers of butan-2-ol (Dickinson & Dalziel, 1967) and octan-2-ol (Van Eys & Kaplan, 1957) and is inactive towards cyclohexanol (Merritt & Tomkins, 1959). There are few data available for either enzyme with regard to their activities with secondary alcohols other than *n*-alkylmethyl carbinols, although it has been reported that hexan-3-ol is a substrate for the liver enzyme (Winer, 1958) and not for the yeast enzyme (Van Eys & Kaplan, 1957). A generalization that appears to explain all these differences of specificity and stereospecificity between the two enzymes is that the yeast enzyme cannot catalyse the oxidation of secondary alcohols in which both alkyl groups are larger than methyl because of steric hindrance, whereas the liver enzyme is free from this restriction (Dickinson & Dalziel, 1967). This hypothesis, based on conclusions with regard to the configuration of substrates and coenzymes in the ternary complexes of both enzymes, was tested further in the present work by studies of their activities towards pentan-3-ol, $(\pm)$ -hexan-3-ol and heptan-4-ol. Initial-rate data for the catalysis of pentan-3-ol oxidation by the liver enzyme are also reported.

MATERIALS AND METHODS

Enzymes and coenzymes. Crystalline alcohol dehydrogenase was prepared from horse liver as described by Dalziel (1961). Yeast alcohol dehydrogenase and NAD⁺ were purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). The NAD⁺ was purified by chromatography on DEAE-cellulose (Dalziel, 1963b).

Alcohols. Pentan-3-ol, $(\pm)$ -hexan-3-ol and heptan-4-ol were from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.) and were fractionally distilled before use. $(\pm)$ -Octan-2-ol (Harrington Bros. Ltd., London, E.C. 1) was purified through the acid phthalate ester as described by Vogel (1956). These four alcohols were at least 95% pure by gas-liquid chromatography. The $(\pm)$ -hexan-3-ol contained approx. 0.05% of ethanol, but this would not affect the stereospecificity studies, and initial-rate studies were not made with this alcohol.

$(\pm)$ -Butan-2-ol was resolved via the brucine salt of the acid phthalate ester. The alcohol was heated with phthalic anhydride and pyridine in equimolar proportions on a water bath for 1 hr. at 50°. The solution was cooled and added to a mixture of 50 ml. of conc. HCl and 500 g. of crushed ice. The ester was extracted with ether (2 × 1 l.), and the combined extracts were washed with 400 ml. of 3*N*-HCl and with distilled water (3 × 800 ml.), dried over MgSO₄ and film-

evaporated. The oily residue was crystallized from ether-light petroleum (b.p. 60–80°) as colourless cubes, m.p. 59°. The ester was treated with an equimolar quantity of brucine in warm acetone, and the $(+)$ -butan-2-ol salt was purified by fractional recrystallization as described by Hofmann, Ess & Usinger (1956). The mother liquors were treated with HCl to remove the brucine and the alcohols were isolated by steam-distillation after addition of 2 equiv. of NaOH. The distillates were dried with K₂CO₃ and fractionally distilled.

The butan-2-ol obtained from the crystalline brucine salt had $[\alpha]_D^{25} + 11.8^\circ$, b.p. 98–100°. Hofmann *et al.* (1956) reported $[\alpha]_D^{25} + 11.6^\circ$, and Norula & Kenyon (1963) found $+13.1^\circ$. The butan-2-ol from the mother liquors, b.p. 98–100°, gave $[\alpha]_D^{25} - 4.0^\circ$, indicating 66% $(-)$ -butan-2-ol. Analytical gas-liquid chromatography revealed 0.05–0.1% of ethanol in both preparations. Neither the commercial $(\pm)$ -butan-2-ol, nor a sample purified via the acid phthalate ester but not resolved, contained ethanol, which was therefore presumably introduced during the resolution. Since even such small amounts of ethanol, a much more active substrate than butan-2-ol, introduce significant errors into the initial-rate parameters, as shown below, the isomers were further purified by preparative gas-liquid chromatography. The ethanol content was thereby decreased less than 0.005%.

Gas-liquid chromatography. Analytical studies were made with a Perkin-Elmer F.11 gas chromatograph, with a column (79 in. × $\frac{1}{8}$ in.) of 15% (w/w) Carbowax 1500 on Chromosorb W (60–80 mesh) at 60° or 100°. The column was supplied and packed by Perkin-Elmer (Beaconsfield, Bucks.) The samples chromatographed were 1 μ l. of 3.3% (v/v) alcohol solution in ether (A.R.). The butan-2-ol isomers were purified in 0.5 ml. portions in an F and M model 770 preparative apparatus at 70°. The column (16 ft. × $\frac{1}{8}$ in.) was 15% (w/w) Carbowax 6000 on Embacel (60–80 mesh). Carbowax 6000 was supplied by Perkin-Elmer, and acid-washed Embacel by May and Baker Ltd. (Dagenham, Essex).

Initial-rate measurements. The recording fluorimeter and technique employed were as described by Dalziel (1962). Reaction mixtures were 10.1 with respect to sodium phosphate buffer, and the temperature was 23.5°. All initial-rate measurements were made in duplicate. Two separate complete experiments were done with $(+)$ -butan-2-ol and with pentan-3-ol. The $(-)$ -butan-2-ol sufficed for only one complete experiment. The kinetic coefficients in the initial-rate equation:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (1)$$

are functions of the rate constants in the mechanism and were evaluated in the normal way from primary and secondary plots of initial-rate data (Dalziel, 1957). In eqn. (1), *e* is the concentration of enzyme active centres, twice the molar concentration; the molecular weight is 84 000 (Ehrenberg & Dalziel, 1958). *S*₁ and *S*₂ are coenzyme and substrate respectively.

Specificity studies. Catalysis of the oxidation of secondary alcohols was tested with large concentrations of both enzymes (up to 4 mg./ml.) and 3.4–4.6 mM-NAD⁺, in glycine buffer, pH 9.5. The alcohol concentrations were 80–90 μ M. No carbonyl-trapping agent was needed as the equilibrium constants for the oxidation of secondary alcohols by NAD⁺

are greater than those for primary alcohols, e.g. $7.2 \times 10^{-9}M$ for propan-2-ol (Burton & Wilson, 1953) and $8.6 \times 10^{-12}M$ for ethanol (Bäcklin, 1958). Progress curves were obtained by measurements of E_{340} with a Zeiss PMQII spectrophotometer; NADH concentrations were calculated from the extinction coefficient $6.22 \times 10^6 \text{ cm}^2/\text{mole}$ (Horecker & Kornberg, 1948). The extinction changes due to endogenous substrate, measured in blanks containing all reactants except secondary alcohol, were subtracted.

Large blanks obtained in early experiments were shown to be caused by substrate in the yeast alcohol dehydrogenase in some commercial NAD⁺ samples and in glass-distilled water. From the rapidity of the blank reaction, and on general grounds, it seemed likely that traces of ethanol were responsible. Chromatographic purification of the NAD⁺,

exhaustive dialysis of the enzyme against buffer solutions in water freshly distilled from alkaline permanganate and the use of such water in the preparation of all reagents decreased the blanks to 0.01–0.02 *E* unit.

It was found incidentally that, though the oxidation of ethanol in small concentrations by NAD⁺ at pH 9.5, catalysed by yeast alcohol dehydrogenase, was incomplete in glycine buffer, as was to be expected from the equilibrium constant, complete oxidation was obtained in tris buffer. The rapid formation of a compound between acetaldehyde and tris was indicated by the disappearance of the absorption maximum of acetaldehyde at $278 m\mu$ on dilution in this buffer, and confirmed the report by Duggan, Donnelly & Melody (1964). Evidently tris is an unsuitable buffer for equilibrium or initial-rate measurements with some carbonyl compounds. It does, however, afford a convenient buffer for the enzymic estimation of small amounts of ethanol without an additional carbonyl-trapping agent (Fig. 1). Liver alcohol dehydrogenase is less satisfactory than the yeast enzyme, because it catalyses a slow oxidation of tris by NAD⁺ (Mahler, 1961).

Fig. 1. Estimation of ethanol with yeast alcohol dehydrogenase. Reaction mixtures contained 0.47M-tris buffer, pH 9.5, 0.44mM-NAD⁺ and 1mg. of yeast alcohol dehydrogenase in a volume of 3ml. NADH formed was estimated from measurements of E_{340} after 10min.

RESULTS

Initial-rate experiments

Average values of the kinetic coefficients in eqn. (1) for the initial rates of oxidation of (+)-butan-2-ol and pentan-3-ol are given in Table 1. The primary and secondary plots of initial-rate data for several substrate and coenzyme concentrations from which these values were obtained (cf. Dalziel & Dickinson, 1966a) were linear over the concentration ranges used. These were: NAD⁺, 7–500 μM; (+)-butan-2-ol, 1–96 mM; pentan-3-ol, 1–72 mM. Also included in Table 1 are kinetic coefficients for (±)-butan-2-ol and propan-2-ol reported previously (Dalziel & Dickinson, 1966a), and kinetic coefficients derived from similar experiments with butan-2-ol isomers, before purification by gas-liquid chromatography, which contained 0.05–0.1% of ethanol. These

Table 1. Kinetic coefficients for the oxidation of secondary alcohols by NAD with liver alcohol dehydrogenase at 23.5° and pH 7.0

The kinetic coefficients are those in the initial-rate eqn. (1) where S_1 is NAD⁺ and S_2 the substrate. The data for (±)-butan-2-ol and propan-2-ol are those of Dalziel & Dickinson (1966a). ϕ_1/ϕ_0 is the K_m for NAD⁺, and ϕ_2/ϕ_0 is the K_m for the substrate.

Substrate	ϕ_0 (sec.)	ϕ_1 (μMsec.)	ϕ_2 (μMsec.)	ϕ_{12} (mM ² sec.)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (μM)	ϕ_{12}/ϕ_2 (μM)
Propan-2-ol	3.9	82	49000	6.2	21	12600	126
(+)-Butan-2-ol	2.3	33	6300	0.48	14.4	2740	76
(±)-Butan-2-ol	1.7	41	9000	0.95	24	5300	105
66% (–)-butan-2-ol	1.6	43	11000	0.75	27	6900	69
(–)-Butan-2-ol*	—	60	18000	1.98	—	—	—
(+)-Butan-2-ol†	1.6	20	3200	0.38	—	2000	—
66% (–)-butan-2-ol†	1.4	21	7100	0.59	—	5050	—
Pentan-3-ol	1.05	23	2500	0.26	22	2480	104

* Calculated (see the text).

† This material contained 0.05–0.1% of ethanol.

Fig. 2. Progress curves for the oxidations of pentan-3-ol (Δ), ($\pm$)-hexan-3-ol ($\bullet$) and heptan-4-ol ($\circ$) by NAD^+ with liver alcohol dehydrogenase at 25° . Reaction mixtures initially contained 33mM-glycine buffer, pH 9.5, 42mM- Na_2HPO_4 , 0.8mg. of liver alcohol dehydrogenase, 4.6mM- NAD^+ and secondary alcohol in a volume of 3ml. The initial concentrations of secondary alcohols were: pentan-3-ol, $87\mu\text{M}$; ($\pm$)-hexan-3-ol, $85\mu\text{M}$; heptan-4-ol, $83\mu\text{M}$. NADH was estimated from measurements of E_{340} .

Fig. 3. Progress curves for the oxidations of ($\pm$)-hexan-3-ol ($\bullet$) and heptan-4-ol ($\circ$) by NAD^+ with liver alcohol dehydrogenase at 25° . Reaction mixtures were initially as described in the legend of Fig. 2 but with 4.8mg. of liver alcohol dehydrogenase.

results show that no significant error would be caused by the trace of ethanol (less than 0.005%) remaining in the purified isomers, but do emphasize the importance of purity of relatively poor substrates if reliable kinetic parameters are to be obtained.

Specificity experiments

Pentan-3-ol, ($\pm$)-*hexan-3-ol* and *heptan-4-ol*. Progress curves for the oxidation of these alcohols by NAD^+ catalysed by liver alcohol dehydrogenase are shown in Fig. 2. Progress curves from a second experiment with ($\pm$)-hexan-3-ol and heptan-4-ol in which six times as much enzyme was used are shown in Fig. 3. Pentan-3-ol is the best of the three substrates, and NADH formation reaches 94% of the theoretical value for complete oxidation of the added alcohol (Fig. 2). With the larger enzyme concentration, ($\pm$)-hexan-3-ol and heptan-4-ol are oxidized to the extent of 84% and 68% respectively in 80min. (Fig. 3). These results show conclusively that all three alcohols are substrates for the liver enzyme and that both isomers of ($\pm$)-hexan-3-ol

Fig. 4. Progress curves for the oxidations of ($\pm$)-butan-2-ol ($\bullet$) and ($\pm$)-octan-2-ol ($\circ$) by NAD^+ with liver alcohol dehydrogenase at 25° . Reaction mixtures initially contained 33mM-glycine buffer, pH 9.5, 34mM- Na_2HPO_4 , 0.3mg. of liver alcohol dehydrogenase, 3.5mM- NAD^+ and secondary alcohol in a volume of 3ml. The initial concentrations of ($\pm$)-butan-2-ol and ($\pm$)-octan-2-ol were $83\mu\text{M}$. The arrow marks the addition of a further 0.3mg. of liver alcohol dehydrogenase.

Fig. 5. Progress curve for the oxidation of (±)-octan-2-ol by NAD^+ with liver alcohol dehydrogenase at 25° . Reaction mixture was initially as described in the legend of Fig. 3 but with 1.8 mg. of liver alcohol dehydrogenase. The arrow marks the addition of a further 1.2 mg. of liver alcohol dehydrogenase.

are oxidized. However, it is evident that one enantiomorph of hexan-3-ol is oxidized more rapidly than the other, since the progress curve in Fig. 3 is biphasic, and with the smaller enzyme concentration in the experiment of Fig. 2 only about half the added alcohol is oxidized in 20 min. The more-active isomer is oxidized at a rate intermediate between those for the shorter-chain and longer-chain alcohols.

In similar experiments with 4 mg. of yeast alcohol dehydrogenase/ml. in place of the liver enzyme, no change in E_{340} was obtained with pentan-3-ol and heptan-4-ol, and with (±)-hexan-3-ol the E_{340} increase in 2 hr. was only 1% of the theoretical for complete oxidation of the added alcohol. These alcohols are therefore not substrates for the yeast enzyme.

(±)-Butan-2-ol and (±)-octan-2-ol. The progress curves obtained with these alcohols (Fig. 4) show that both isomers are substrates for liver alcohol dehydrogenase. There is no evidence of a gross difference in the rate of oxidation of the butan-2-ol isomers, in accordance with the similarity of the initial-rate parameters in Table 1. However, the biphasic nature of the progress curve for (±)-

Fig. 6. Progress curves for the oxidations of (±)-butan-2-ol (●) and (±)-octan-2-ol (○) by NAD^+ with yeast alcohol dehydrogenase. Reaction mixtures initially contained 31 mM-glycine buffer, pH 9.5, 32 mM- Na_2HPO_4 , 11 mg. of yeast alcohol dehydrogenase, 3.3 mM- NAD^+ and secondary alcohol in a volume of 3 ml. The initial concentrations of (±)-butan-2-ol and (±)-octan-2-ol were $78 \mu\text{M}$. The arrow marks the addition of a further 4.5 mg. of yeast alcohol dehydrogenase.

octan-2-ol oxidation shows that one enantiomorph is oxidized much more rapidly than the other, and also more rapidly than the butan-2-ol isomers. This is confirmed by an experiment in which six times as much enzyme was used (Fig. 5).

Similar experiments with yeast alcohol dehydrogenase (Fig. 6) confirm previous observations that only one enantiomorph of each of octan-2-ol (Van Eys & Kaplan, 1957) and of butan-2-ol (Dickinson & Dalziel, 1967) is a substrate of this enzyme, and show that the active isomer of octan-2-ol is oxidized more rapidly than that of butan-2-ol, as with the liver enzyme, under these conditions of low substrate concentration and saturating coenzyme concentration.

DISCUSSION

Liver alcohol dehydrogenase does not exhibit absolute stereospecificity towards the optically active substrates butan-2-ol, octan-2-ol and hexan-3-ol. Whereas the enantiomorphs of butan-2-ol react at rather similar rates, there is a considerable difference in this respect between the isomers of the two higher alcohols, in each case with low substrate concentrations and saturating coenzyme concentration (Figs. 2-5). No initial-rate studies were made with the higher alcohols, but it can be concluded from these findings that values for ϕ_2 in eqn. (1) must be very much smaller for the more-active than for the less-active enantiomorphs. Initial-rate studies with pure (+)-butan-2-ol and partially resolved (-)-butan-2-ol show directly that ϕ_2 is

smaller for the (+)-isomer (Table 1), but the difference is not large enough to result in clearly biphasic progress curves in the oxidation of the racemic alcohol, as is observed with the higher alcohols. In contrast with these results with the liver enzyme, it is confirmed that yeast alcohol dehydrogenase exhibits absolute stereospecificity for the (+)-isomers of octan-2-ol (Van Eys & Kaplan, 1957) and butan-2-ol (Dickinson & Dalziel, 1967).

It has been suggested (Dickinson & Dalziel, 1967) that the (+)-isomers of *n*-alkylmethyl carbinols form active ternary complexes by combining with the NAD^+ complexes of both enzymes through hydrophobic bonding of the larger alkyl group. With a second binding site specific for the hydroxyl group, the requisite orientation of the hydrogen atom with respect to the coenzyme can be achieved. If the (–)-isomers are to be oriented in an active configuration in the ternary complex of the liver enzyme, the methyl group must be attached to the hydrophobic site. These conclusions, which differ with regard to the yeast enzyme from those of Van Eys & Kaplan (1957), were based on the following facts.

(1) The configurations of the enantiomorphs of butan-2-ol (Levene, Walti & Haller, 1926) and higher *n*-alkylmethyl carbinols (Levene & Marker, 1932; Levene & Harris, 1936; Mills & Klyne, 1954) are known: the (+)-isomers all have the *S*-configuration in the nomenclature of Cahn, Ingold & Prelog (1956).

(2) The isomers of $[1\text{-}^3\text{H}]\text{ethanol}$ (Levy, Loewus & Vennesland, 1957; Lemieux & Howard, 1963) and that of $[1\text{-}^3\text{H}]\text{geraniol}$ (Donninger & Ryback, 1964), from which isotope is specifically transferred to NAD^+ in the oxidations catalysed by yeast and liver alcohol dehydrogenase respectively, both have the *R*-configuration.

(3) The A-specific reduction of NAD^+ is catalysed by the yeast enzyme with both ethanol and propan-2-ol as substrates (Levy & Vennesland, 1957), and by the liver enzyme with ethanol (Levy & Vennesland, 1957) and also propan-2-ol and both isomers of butan-2-ol (Dickinson & Dalziel, 1967) as substrates. The configurations of these substrates, and their suggested orientations in the ternary complexes of the two enzymes, are shown diagrammatically in Fig. 7.

The initial-rate parameters for (+)-butan-2-ol, 66% (–)-butan-2-ol and (±)-butan-2-ol (Table 1) provide some further support for these conclusions with regard to the liver enzyme. It has been shown previously that, with primary alcohols, ϕ_2 and K_m (i.e. ϕ_2/ϕ_0) decrease with increase of alkyl-chain length from ethanol to butanol, suggesting that the affinity of the hydrophobic site for the alkyl group increases with the size of the latter (Dalziel &

Fig. 7. Configurations and orientations in the ternary complexes of yeast and liver alcohol dehydrogenases of the enantiomorphs of *n*-alkylmethyl carbinols and $[1\text{-}^3\text{H}]\text{ethanol}$. An alkyl site (a) and hydroxyl site (b) on the enzyme are shown. For (+)-2*S*-*n*-alkylmethyl carbinols, which are substrates of both enzymes, R is *n*-alkyl and R' is methyl. For (–)-2*R*-*n*-alkylmethyl carbinols, which are substrates of the liver enzyme only, R is methyl and R' is *n*-alkyl. For (–)-1*S*- $[1\text{-}^3\text{H}]\text{ethanol}$, from which ^3H is not transferred to NAD^+ , R is methyl and R' is ^3H . The nicotinamide ring is represented in accordance with the A-specific reduction of NAD^+ and the absolute configuration determined by Cornforth, Ryback, Popják, Donninger & Schroeffer (1962). For other references see the text.

Dickinson, 1966a). The conclusion that the ethyl group of (+)-butan-2-ol and the methyl group of (–)-butan-2-ol are bound at the hydrophobic site is thus consistent with the ϕ_2 values in Table 1. Although the experiments with octan-2-ol were confined to the racemic mixture, it can be assumed on stereochemical grounds that the (+)-isomer is the more active one in this case also, and the fact that it is much more active than (+)-butan-2-ol is also in line with the effect of alkyl-chain length on ϕ_2 . It has been shown experimentally that the (+)-isomers of butan-2-ol (Dickinson & Dalziel, 1967) and octan-2-ol (Van Eys & Kaplan, 1957) are the active forms with the yeast enzyme, and again the latter is more active than the former (Fig. 6), but kinetic data for this enzyme with primary and secondary alcohols are not yet available in sufficient detail to permit any such generalization about the effect of alkyl-chain length.

Most dehydrogenases show absolute stereospecificity towards their optically active substrates, which generally have two different unreactive groups attached to the asymmetric carbon atom, e.g. methyl and carboxyl groups in lactate. The unusual lack of such specificity by liver alcohol dehydrogenase for secondary alcohols may be regarded as the expected consequence of the facts that both groups are simple alkyl and that the hydrophobic site can combine with a range of alkyl groups, as shown by the reactivity of a variety of

primary and secondary alcohols. The same considerations apply to the yeast enzyme, however, yet it exhibits absolute stereospecificity for the (+)-isomers of butan-2-ol and octan-2-ol. To explain this, it was suggested that, though the liver enzyme can accommodate an alkyl group larger than methyl not bound at the hydrophobic site (R' in Fig. 7), as is required for reactive orientation of the (-)-isomers, the yeast enzyme cannot (Dickinson & Dalziel, 1967). This implies that secondary alcohols other than *n*-alkylmethyl carbinols should be substrates of the liver enzyme, but not of the yeast enzyme. There were reports that this was true of hexan-3-ol (Winer, 1958; Van Eys & Kaplan, 1957), and cyclohexanol (Merritt & Tomkins, 1959) might be regarded as another example. The hypothesis receives strong support from the present findings that pentan-3-ol and heptan-4-ol, as well as hexan-3-ol, are substrates for the liver enzyme but not for the yeast enzyme. It should be added that no experimental evidence is yet available to show whether these alcohols, and the (-)-isomers of *n*-alkylmethyl carbinols, are actually unable to combine with the NAD^+ complex of the yeast enzyme, or whether steric hindrance instead prevents a conformational change that might follow substrate combination and be essential for reaction within the ternary complex.

The results indicate a fundamental difference between the active centres of the two enzymes. The amino acid sequences of the two proteins near the active centres are known to differ significantly (Harris, 1964), but their differences of specificity probably also reflect differences of secondary and tertiary structure, and the evidence presented here with regard to the orientation and binding of the various substrates may help in their elucidation. It should be pointed out that the liver enzyme does show some degree of stereospecificity, which increases as the size of the unbound alkyl group increases: the difference between the rates of oxidation of the enantiomorphs increases in the sequence butan-2-ol, hexan-3-ol and octan-2-ol (Figs. 2-5). Also, heptan-4-ol is a poorer substrate than the more active isomer of hexan-3-ol (Fig. 2).

The initial-rate parameters for (+)-butan-2-ol and pentan-3-ol are consistent with the mechanism previously suggested for liver alcohol dehydrogenase (Dalziel & Dickinson, 1966a) and need not be discussed in detail. The ratios ϕ_{12}/ϕ_2 , which should be independent of the nature of the substrate and equal to the dissociation constant of $E \cdot NAD^+$, are in satisfactory agreement with those obtained for propan-2-ol and ($\pm$)-butan-2-ol, cyclohexanol and several primary alcohols, and for aldehydes in the aldehyde-dehydrogenase reaction. The ratios ϕ_1/ϕ_0 (K_m for the coenzyme) also agree reasonably well with those obtained for other secondary alcohols and

aldehydes, in accordance with the interpretation suggested previously.

Experimental initial-rate parameters are not available for pure (-)-butan-2-ol, but can be calculated from initial-rate data for either ($\pm$)-butan-2-ol or 66% (-)-butan-2-ol, together with the parameters for pure (+)-butan-2-ol, on the basis of the proposed mechanism. Consistent values are obtained, shown in Table 1. This calculation does not permit an estimate of ϕ_0 , but it is obvious from Table 1 that it is a little smaller than for the (+)-isomer.

It is not possible at present to account for the relative reactivities of all these substrates of liver alcohol dehydrogenase. Factors other than steric ones and the varying affinity of alkyl groups for the hydrophobic site may be important, for example the varying acid character of the hydroxyl group. The greater acid strength of primary alcohols compared with secondary alcohols (Hine & Hine, 1952; Kuhn, 1952) may be one reason why the former are the better substrates and are oxidized more rapidly within the ternary complex, as indicated by the greater maximum rate ($1/\phi_0$) (Dalziel & Dickinson, 1966a). Primary alcohols all give the same maximum rate, which is determined by the rate of dissociation of the product complex, $E \cdot NADH$. Secondary alcohols give different maximum rates, indicating that the rate of oxidation within the ternary complex is rate-limiting. From the increase of maximum rate in the sequence propan-2-ol, butan-2-ol and pentan-3-ol, it appears that the rate of oxidation within the ternary complex increases with size of both alkyl groups of secondary alcohols. Unfortunately, higher secondary alcohols are too insoluble to permit accurate evaluation of their initial-rate parameters.

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