Enzymes in Organic Synthesis. Influence of Substrate Structure on Rates of Horse Liver Alcohol Dehydrogenase-catalysed Oxidoreductions

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Detailed rate studies on horse liver alcohol dehydrogenase (HLADH)-catalysed oxidoreductions of a broad structural range of aliphatic alcohol and carbonyl substrates of organic chemical interest have been carried out in order to identify kinetic factors which remain significant in preparative-scale asymmetric synthetic applications of the enzyme. The data are consistent with an ordered Theorell–Chance mechanism for all the substrates examined. Coenzyme dissociation is largely rate-determining for primary alcohol and aldehyde oxidoreductions, but not for secondary alcohols or ketones. To a considerable extent hydrophobic binding of a substrate at the active site can be correlated with its relative reactivity. The preparative-scale implications of these results are discussed. One of the more important conclusions is that the degree of enantioselectivity achievable during HLADH-mediated transformations of racemates can be manipulated in some cases by varying the substrate concentration.

OXIDOREDUCTIONS of the C=O $\rightleftharpoons$ CH(OH) type are of central importance in synthetic organic chemistry. Such reactions are catalysed by alcohol dehydrogenases and the asymmetric synthesis potential of these enzymes has now been documented for a wide range of substrates possessing structures of organic chemical rather than biochemical interest.[1] The alcohol dehydrogenase from horse liver (EC 1.1.1.1, HLADH †) is the best documented enzyme in this regard.[1-3] A minimal mechanism for HLADH-catalysed oxidoreductions is the ordered Theorell–Chance pathway.[4a] This is represented in equation (1). For many of the alcohol and carbonyl substrates for which kinetic data are available, binding of coenzyme and substrate occur sequentially as shown. Furthermore, dissociation of the nicotinamide coenzyme, NAD^+ or NADH, is often rate determining for structurally simple aldehyde and primary alcohol substrates.[1,4] For such compounds therefore, the maximum velocity of the HLADH-mediated reaction is unaffected by the structure of the substrate.

The implications of limiting conditions of this type with respect to facilitating the planning of preparative scale reactions, for example in matters such as estimating reaction times, are self evident. Accordingly, as part

$$E + NAD^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.NAD^+ + S_{OH} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E.NAD^+.S_{OH} \underset{k'}{\overset{k}{\rightleftharpoons}} E.NADH.S_{CO} \underset{k_2'}{\overset{k_{-2}'}{\rightleftharpoons}} S_{CO} + E.NADH \underset{k_1'}{\overset{k_{-1}'}{\rightleftharpoons}} E + NADH \quad (1)$$

† Abbreviations used: HLADH, horse liver alcohol dehydrogenase; NAD^+/NADH, nicotinamide adenine dinucleotide, oxidised and reduced forms respectively; S_{CO}, carbonyl substrate; S_{OH}, alcohol substrate.

[1] J. B. Jones and J. F. Beck, 'Applications of Biochemical Systems in Organic Chemistry,' eds. J. B. Jones, C. J. Sih, and D. Perlman, Wiley, New York, 1976, part I, ch. 4.

[2] A. J. Irwin and J. B. Jones, *J. Amer. Chem. Soc.*, (*a*) 1976 **98**, 8476; 1977, **99**, (*b*) 556; (*c*) 1625.

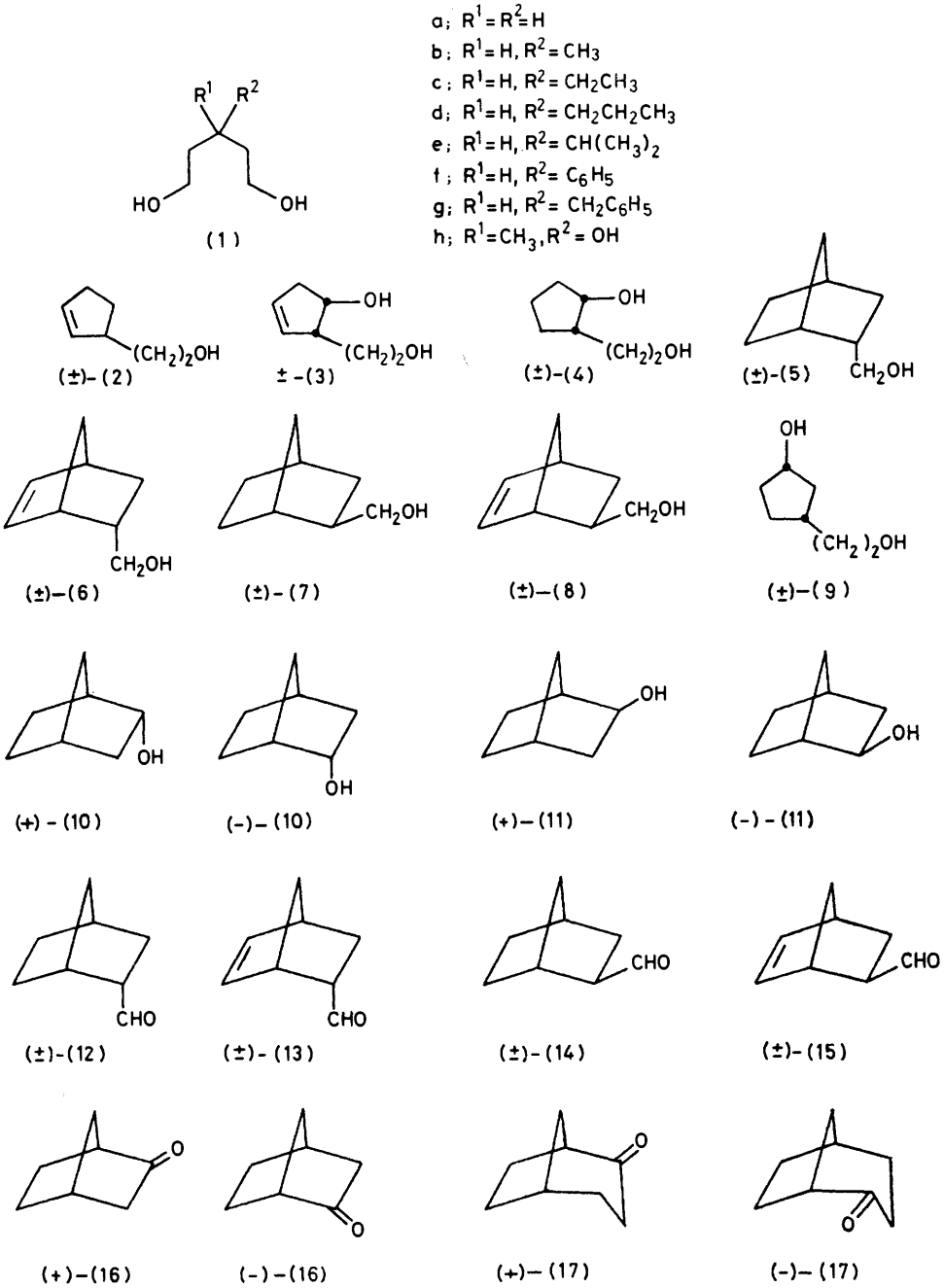
[3] J. B. Jones and H. B. Goodbrand (*a*) *Canad. J. Chem.*, 1977, **55**, 2685; (*b*) *J.C.S. Chem. Comm.*, 1977, 469.

[4] K. Dalziel, (*a*) ' The Enzymes ' eds. P. D. Boyer, Academic Press, New York, 1975, 3rd edn., vol. 11, pp. 1—60; *Acta Chem. Scand.*, 1957, **11**, (*b*) 1706; (*c*) 397.

of a systematic delineation of the structural range for which the rate-limiting enzyme–coenzyme dissociation condition holds, we have examined the rate of HLADH-catalysed oxidoreductions of the substrates (1)—(17) for

RESULTS

The substrates were purchased or were prepared by literature methods or by unexceptional alternative routes. The kinetics were followed at pH 9 for oxidation and at



which preparative scale reactions have already proven of asymmetric synthesis value. The results obtained show that enzyme–coenzyme dissociation can remain rate determining over a remarkable variation in substrate structure. The data also confirm the importance of hydrophobic interactions between enzyme and substrate.

pH 7 for reduction using saturating concentrations of NAD^+ (5×10^{-4}M) and NADH (1.75×10^{-4}M) respectively. Care was taken to ensure that no substrate activation or inhibition occurred under the assay conditions. Linear Lineweaver–Burk plots were obtained for each substrate at concentrations up to the following maximum levels: (1), 2×10^{-2}M; ±-(2)—±-(6), ±-(8)—±-(11), ±-(16),

±-(17), 1×10^{-2}M; −-(10), 2×10^{-3}M; ±-(7), ±-(12)—±-(15), *ca.* 5×10^{-4}M. The kinetic constants for the reference compounds (±)-3-methylhexanol, cyclohexanol, cyclopentanol, and cyclohexanone were similarly determined on solutions whose maximum concentrations were $\leqslant 10^{-2}$, 10^{-2}, 10^{-3}, and 10^{-2}M, respectively. Substrate inhibition was observed at concentrations of $\geqslant$4mM for ±-(5), $\geqslant$2mM for ±-(6), $\geqslant$0.25mM for ±-(7), and $\geqslant$1mM for cyclopentanol and substrate activation at $\geqslant$8mM for cyclohexanol. The data obtained are summarized in Tables 1—3, largely in the ϕ parameter form devised by Dalziel.[4]

DISCUSSION

Dalziel [4] found that HLADH-catalysed oxidations obeyed an equation of the form (2) where e = concen-

$$e/v_0 = \phi_0 + \phi_1/[NAD^+] + \phi_2/[S_{OH}] + \phi_{12}/[NADH][S_{OH}] \quad (2)$$

tration of enzyme active sites and v_0 = initial velocity. The equation for reductions is analogous, with ϕ' parameters used to denote the reverse direction. Reporting kinetic data in ϕ parameter form has many advantages.[4-6] Furthermore, once ϕ_0 and ϕ_2 have been determined, the more traditional maximum velocity ($V_{max.}$) and Michaelis constant (K_m) descriptors for the substrates are easily obtained from the relationships $V_{max.} = 1/\phi_0$ and $K_m(S_{OH}$ or $S_{CO}) = \phi_2/\phi_0$. The determination of true ϕ_0 and ϕ_2 values for a substrate is not a trivial matter, requiring as it does multiple kinetic runs at several substrate and coenzyme concentrations.[4-6] Accordingly, in view of the large number of substrates being evaluated, we elected to use the more readily obtainable apparent parameters ϕ_0(app.) and ϕ_2(app.) where $\phi_0(app.) = \phi_0 + \phi_1/[coenzyme]$ and $\phi_2(app.) = \phi_2 + \phi_{12}/[coenzyme]$. For all substrates examined previously, ϕ_1 and ϕ_{12} values are small.[4-6] Real and apparent ϕ_0 parameters thus become negligibly different at high coenzyme concentrations. From the published ϕ_0 and ϕ_1 data,[4-6] we calculated that using $[NAD^+] \geqslant 5 \times 10^{-4}$M or $[NADH] \geqslant 1.75 \times 10^{-4}$M would assure this limiting condition.*

Under these circumstances, the apparent Michaelis constants thus become effectively equal to the true values.† In fact the differences between K_m(true) and K_m(app.) are of the same order of magnitude as the overall experimental errors involved in the actual ϕ parameter determinations. All kinetic runs were therefore carried out at the fixed coenzyme concentrations of 5×10^{-4}M for oxidations and 1.75×10^{-4}M for reductions and the apparent ϕ and K_m values obtained taken as reflecting closely those of the true coefficients.

The kinetic data are generally consistent with a compulsory order mechanism of the type in equation (1) for each of the substrates (1)—(17) under the assay conditions applied. Substrate activation or inhibition was not a major problem, being observed only for six compounds. The activation noted at the higher substrate concentrations for ±-(8) and cyclohexanol, and the inhibition for ±(5)—±-(7) and cyclopentanol, are adequately accommodated by invoking deviations from the equation (1) pathway owing to formation of abortive $E.NADH.S_{OH}$ and active $E.S_{OH}$ complexes respectively.[4a,6-8] For the most hydrophobic of the substrates studied, the upper concentration limits were set by solubility factors rather than by considerations of possible substrate activation or inhibition.

With ±-(2) and ±-(8) as the only two exceptions, the primary alcohols of Table 1 all have effectively the same ϕ_0(app.) parameters. The values are virtually identical with ϕ_0 for ethanol and cyclohexanol. The final NAD^+ dissociation step is thus rate determining in all these cases. Considering the structural and enantiomeric variations of the Table 1 substrates, this is a remarkable observation. It is of great practical significance since it means that primary alcohols of quite diverse structures can be expected to undergo HLADH-catalysed oxidations with the same maximum velocity.

As Table 2 shows, the situation for the secondary alcohols surveyed is quite different. Only for ±-(9) and ±-(10) are the values of ϕ_0(app.) within the 0.3—0.4 range required for rate-determining coenzyme dissociation.[4-6] For all others the magnitudes of ϕ_0(app.) are significantly higher than 0.4, thereby indicating that hydride transfer is at least partly rate limiting in these cases.[4-10] It is thus evident that, at present, maximum velocities of oxidation cannot be predicted with any confidence for alicyclic secondary alcohols ‡ since the rate variations can be very great, even within a structurally similar series. For example, the ϕ_0(app.) ($= 1/V_{max.}$) values for (2*R*)-norbornan-*endo*-2-ol +-(10) and (2*R*)-norbornan-*exo*-2-ol —-(11) differ by two orders of magnitude.

For simple aliphatic aldehydes, NAD^+ dissociation in

* Based on literature ϕ_2 and ϕ_{12} data,[5,6] ϕ_2 (app.) values at these limiting coenzyme levels are consistently higher than the true ϕ_2 coefficients by only 20 ± 5% for alcohol oxidations and 2 ± 1% for carbonyl reductions. Furthermore, individual ϕ_2 values have little significance. It is only a series of ϕ_2 values that can be used to interpret the effectiveness of substrate binding. A series of ϕ_2 (app.) coefficients is thus equally valuble in this regard, provided that the systematic error remains fairly constant.

† It is of interest to note that most literature [1] $K_m(S_{CO}$ or $S_{OH})$ values are apparent Michaelis constants only since the majority were determined at a single coenzyme concentration.

‡ Attention is drawn to the fact that cyclopentanol undergoes ready oxidation. The earlier report [11] that it was a non-substrate may have been the consequence of its having been assayed at high concentrations. Substrate inhibition would be severe under such conditions.

[5] C. S. Tsai, *Canad. J. Biochem.*, 1968, **46**, 381.

[6] K. Dalziel and F. M. Dickinson, *Biochem. J.*, 1966, **100**, (*a*) 34; (*b*) 491; (*c*) F. M. Dickinson and K. Dalziel, *ibid.*, 1967, **104**, 165.

[7] (*a*) C. S. Hanes, P. M. Bronskill, P. A. Gurr, and J. T-F. Wong, *Canad. J. Biochem.*, 1972, **50**, 1385; (*b*) P. A. Gurr, P. M. Bronskill, C. S. Hanes, and J. T-F. Wong, *ibid.*, p. 1376.

[8] E. Silverstein and P. D. Boyer, *J. Biol. Chem.*, 1964, **239**, 3908.

[9] G. R. Ainslie and W. W. Cleland, *J. Biol. Chem.*, 1972, **247**, 946.

[10] R. L. Brooks and J. D. Shore, *Biochemistry*, 1971, **10**, 3855; R. L. Brooks, J. D. Shore, and H. Gutfreund, *J. Biol. Chem.*, 1972, **247**, 2382.

[11] A. D. Merritt and G. M. Tomkins, *J. Biol. Chem.*, 1959, **234**, 2778.

the terminal step of equation (1) is rate limiting.[4,6] The results in Table 3 show that this step can still be largely rate determining even when the aldehydes are structurally much more complex. The data for the sociation. It may be that hydride transfer has become rate limiting, at least in part, for these substrates. Alternatively, since the product alcohols are very hydrophobic, their dissociation from the ternary complex

TABLE 1

Kinetic parameters for HLADH-catalysed oxidations of the primary alcohols (1)—(8) [a]

Substrate	K_m(app.)/mmol l^{-1}	ϕ_0(app.)/s	ϕ_2(app.)/ μmol s l^{-1}	$10^3\phi_2$(app.)$^{-1}$/ l mol^{-1} s^{-1}
(1a)	0.66	0.32	212	4.7
(1b)	0.50	0.36	180	5.6
(1c)	0.71	0.44	310	3.2
(1d)	0.11	0.44	48.4	20.7
(1e)	0.22	0.52	114	8.8
(1f)	3.0	0.42	1 260	0.8
(1g)	1.67	0.58	967	1.0
(1h)	24.0	0.56	13 400	0.07
(±)-3-Methylhexanol	19.0	0.38	7 300	0.14
±-(2)	0.22	1.72	370	2.7
±-(3)	6.7	0.31	2 100	0.5
±-(4)	16.0	0.38	6 200	0.2
±-(5)	0.19	0.31	57	17.5
±-(6)	0.15	0.41	61	16.4
±-(7)	0.04	0.67	28	35.7
±-(8)	0.01	1.20	17	58.8
CH_3CH_2OH [b]	0.70 [d]	0.42 [d]	294 [d]	3.4
Cyclohexanol	0.90	0.30	266	3.8
Cyclohexanol [c]	0.74 [d]	0.31 [d]	230 [d]	4.3

[a] Determined at 25 °C in 0.05M-glycine–NaOH buffer, pH 9 with [NAD^+] 5.0 × 10^{-4}M and [S_{OH}] 10^{-4}—10^{-2}M. [b] Ref. 5. [c] Ref. 6*b*; pH 7. [d] True values.

TABLE 2

Kinetic parameters for HLADH-catalysed oxidations of the secondary alcohols (9)—(11) [a]

Substrate	K_m(app.)/mmol l^{-1}	ϕ_0(app.)/s	ϕ_2(app.)/ μmol s l^{-1}	$10^3\phi_2$(app.)$^{-1}$/ l mol^{-1} s^{-1}
Cyclopentanol	0.13	1.23	160	6.3
±-(9)	3.6	0.38	1 400	0.7
±-(10)	0.10	0.38	39	25.6
−-(10) [b]	0.35	1.13	390	2.6
+-(11)	2.40	4.25	10 000	0.1
−-(11)	31.0	37.0	1.1 × 10^6	0.0009

[a] Determined at 25 °C in 0.05M-glycine–NaOH buffer, pH 9 with [NAD^+] 5.0 × 10^{-4}M and [S_{OH}] 10^{-4}—10^{-2}M. [b] Maximum concentration 2.5 × 10^{-4}M.

TABLE 3

Kinetic parameters of HLADH-catalysed reductions of the carbonyl substrates (12)—(17) [a]

Substrate	K_m(app.)/mmol l^{-1}	ϕ_0'(app.)/s	ϕ_2'(app.)/ μmol s l^{-1}	$10^3\phi_2'$(app.)$^{-1}$/ l mol^{-1} s^{-1}
Acetaldehyde [d]	0.44 [e]	0.008 [e]	3.3 [e]	303
Cyclohexanone	16.5	0.018	298	
Cyclohexanone [d]	16.8 [e]	0.017 [e]	285 [e]	3.5
±-(12) [b]	0.23	0.013	3.0	333
±-(13) [b]	0.30	0.017	5.0	200
±-(14) [b]	0.19	0.009	1.7	590
±-(15) [b]	0.09	0.020	1.8	555
+-(16) [c]	3.1	0.12	370	2.7
−-(16) [c]	2.9	2.39	7 000	0.1
+-(17) [c]	8.1	0.035	280	3.6
−-(17) [c]	43.5	0.13	5 650	0.18

[a] Determined at 25 °C in 0.1M-potassium phosphate buffer, pH 7 with [NADH] 1.75 × 10^{-4}M. [b] [S_{CO}] ⩽ 10^{-4}M. [c] [S_{CO} 10^{-4}—10^{-2}M. [d] Ref. 6*b*. [e] True values.

bicyclic substrates (12)—(15) illustrate this point. In contrast to aldehydes ketones are, in general, rather poor substrates of HLADH [1] and those of Table 3 are no exception. The ϕ_0'(app.) values of the bridged bicyclic ketones (16) and (17) (Table 3) are significantly higher than that corresponding to rate-limiting E.NAD^+ dis- might be the slow step, as it is with aromatic alcohols.[12] This is clearly a very structure sensitive factor with $V_{max.}$ variations of almost 10^2-fold manifest even within the family of substrates (16) and (17).

While the K_m(app.) constants recorded in Tables 1—3 are not Michaelis constants as usually defined,[13] they are

[12] S. A. Bernhard, M. F. Dunn, P. L. Luisi, and P. Schack, *Biochemistry*, 1970, **9**, 185; J. T. McFarland and S. Bernhard, *ibid.*, 1972, **11**, 1486.

[13] M. Dixon and E-C. Webb, ' Enzymes,' Longmans, London, 1964, 2nd edn., p. 65.

considered to reflect the relative order of binding of the substrates, particularly within a structurally related series. The overall K_m(app.) data confirm that the substrate binding site is hydrophobic in character,[1,14] with the most non-polar substrates generally binding most strongly. However, steric factors must also be taken into account. For example, the diol (1f), with a bulky 3-phenyl group, is more hydrophobic than its unsubstituted parent (1a) but binds five times less strongly to the enzyme. One must also be cautious in interpreting the effect of hydrophilic groups. From a comparison of the K_m(app.) constants of the diols ±-(3) and ±-(4) with that of their parent primary alcohol ±-(2) it is seen that the secondary alcohol functions of (3) and (4) exert a deleterious effect on binding. This can be attributed to the fact that binding of the two diols requires the polar CH(OH) groups to locate in a hydrophobic region of the active site.[2c] On the other hand, for 3-hydroxy-3-methylpentane-1,5-diol (1h) the effect of the 3-hydroxy group on K_m has a steric basis only.[2b]

Tsai [5] found $1/\phi_2$ to be a useful measure of hydrophobic binding and of the relative reactivities of substrates. The relative magnitudes of $1/\phi_2$(app.) for the substrates in this study also follow the general relative reactivity order observed for (1)—(17) in the previous preparative-scale reactions.[2] Furthermore, for those substrates having the same ϕ_0(app.) values, the magnitudes of $1/\phi_2$(app.) reflect the degree of hydrophobic binding. Such criteria of relative reactivity are of considerable practical significance since maximal oxidoreduction rates will seldom be attainable in preparative scale reactions with hydrophobic substrates because of solubility difficulties.

For racemic substrates, differences in reactivity between enantiomers are superimposed on the general structural considerations outlined above. This is of primary importance with respect to asymmetric synthetic applications of HLADH. Individual enantiomers can have markedly different ϕ_0(app.) and K_m(app.) values (Tables 2 and 3) and the degrees of enantioselectivities observed in preparative scale oxidoreductions of ±-(11), ±-(16), and ±-(17) [2a] are in good accord with these kinetic data. However, for many of the racemic substrates in this study the ϕ_0(app.) values are essentially equal to the ϕ_0 value for ethanol. In such cases, coenzyme dissociation is evidently rate limiting for *each* stereoisomer. As a consequence, no enzymic discrimination between enantiomers will occur if such racemates are oxidoreduced under $V_{max.}$ (high substrate–low enzyme concentrations) conditions. Fortunately, from the point of view of HLADH-mediated resolutions, enzymic discrimination between enantiomers having the same $V_{max.}$ is not totally precluded. Such distinctions can be induced by operating at substrate concentrations sufficiently below those required for maximal rates that any differences in active-site interactions between individual stereoisomers are not over-ridden. The enantioselectivities observed in the preparative scale HLADH-catalysed oxidations of ±-(3) and ±-(9) attest to the practicality of this concept.[2c] From this analysis it also follows that different degrees of stereospecificity can be expected at different substrate concentrations. Accordingly, whenever a racemate is being subjected to HLADH-mediated oxidoreduction for asymmetric synthesis or resolution purposes, the substrate concentration should be varied in order to establish the optimum level for the enantioselectivity desired.

EXPERIMENTAL

Unless indicated otherwise, sources of materials, equipment used, analytical procedures, criteria of purity, *etc.*, were as described previously.[2]

Preparation of Substrates.—The alcohols (1b, e, f, h), ±-(2)—±-(4), ±-(9), ±-(10), −-(10), +-(11), and −-(11) and the ketones +-(16), −-(16), +-(17), and −-(17) were prepared in earlier work.[2] *endo*-±-(6) and *exo*-2-hydroxymethylnorborn-5-ene ±-(8) and the corresponding norbornanes ±-(5) and ±-(6) were obtained by the procedure of Berson and Walia.[15] *endo*-±-(13) and *exo*-2-formylnorborn-5-ene ±-(15) were obtained by spinning band distillation of the commercially available (Aldrich) *endo*–*exo* mixture. Hydrogenation of ±-(13) and ±-(15) to the 2-formylnorbornanes ±-(12) and ±-(14) respectively was effected as described by Freeman and Desai.[16] The diol (1a) was purchased from Aldrich. Acetaldehyde, ethanol, cyclohexanol, and cyclopentanol were redistilled prior to use.

(±)-3-*Methylhexanol.* The general procedure of Knights and Brown [17] was used. 3-Methylhex-1-ene (9.8 g, 0.1 mol; Aldrich) in dry tetrahydrofuran (50 ml) was added slowly under nitrogen at 25 °C *via* a syringe to 9-borabicyclo[3.3.1]nonane in dry tetrahydrofuran (0.5M, 400 ml; Aldrich). The mixture was stirred for 1 h and the reaction then quenched with 6M aqueous sodium hydroxide (50 ml). 30% Hydrogen peroxide (40 ml) was then added dropwise and the mixture heated at 60 °C for 2 h. It was then cooled and poured into water (600 ml) and extracted with diethyl ether (2 × 150 ml). The ether extract was washed with saturated aqueous sodium chloride, dried ($MgSO_4$), rotary evaporated, and distilled to give (±)-3-methylhexanol (9 g, 78%), b.p. 88 °C at 25 mmHg (lit.,[18] 80 °C at 25 mmHg).

3-*Ethylpentane*-1,5-*diol* (1c). Diethyl ethylmalonate (130 g, 0.69 mol; prepared by the method of Adams and Kamm [19]) in dry diethyl ether (500 ml) was added to lithium aluminium hydride (26.6 g, 0.7 mol) in dry diethyl ether (800 ml). The mixture was refluxed overnight and then quenched by the careful addition of water (80 ml) followed by 15% aqueous sodium hydroxide (27 ml). The resulting mixture was filtered and the residue washed with diethyl ether (100 ml). The combined filtrate and washings were dried ($MgSO_4$), rotary evaporated, and distilled to

[14] C-I. Bränden, H. Jörnvall, H. Eklund, and B. Furigen in ref. 4*a*, pp. 107—171

[15] J. A. Berson and J. G. Walia, *J. Amer. Chem. Soc.*, 1961, **83**, 3986.

[16] P. K. Freeman and K. B. Desai, *J. Org. Chem.*, 1971, **36**, 1554.

[17] E. F. Knights and H. C. Brown, *J. Amer. Chem. Soc.*, 1968, **90**, 5283.

[18] A. Dewael and A. Weckering, *Bull. Soc. chim. Belges*, 1924, **33**, 495.

[19] R. Adams and R. M. Kamm, *Org. Synth.*, 1948, Coll. Vol. 1, 250.

give 2-ethylpropane-1,3-diol (66.4 g, 72%), b.p. 115—116 °C at 1.3 mmHg.

A solution of this diol (60 g, 0.58 mol) in dry benzene (600 ml) and dry pyridine (180 ml) was cooled (5 °C) and methanesulphonyl chloride (136.8 g, 1.2 mol) added over 30 min at 5 °C. The mixture was stirred at 20 °C for 1 day, filtered, and the precipitate washed with benzene (100 ml). The combined filtrate was washed with saturated aqueous sodium chloride (100 ml). The benzene layer was then decolourised with charcoal, dried ($MgSO_4$), and rotary evaporated to yield 2-ethylpropane-1,3-diyl bismethanesulphonate (130.9 g, 87%). This was dissolved in ethanol (800 ml) and a solution of potassium cyanide (65.9 g, 1 mol) in water (400 ml) added. The mixture was refluxed for 6 h. 10N Aqueous sodium hydroxide (400 ml) was added and refluxing was continued for a further 1 day. The mixture was concentrated by distilling off ethanol (350 ml); it was then washed with diethyl ether. The aqueous layer was decolourised with charcoal, cooled (0 °C), and acidified slowly with concentrated hydrochloric acid (400 ml). The resulting solution was washed with several portions of diethyl ether, then decolourised with charcoal once again and concentrated. On keeping the residue at −10 °C, 3-ethylglutaric acid was obtained as crystals (31.3 g, 39%), m.p. 75—76 °C (lit.,[20] 73 °C), δ[$(CD_3)_2SO$] 0.9 (3 H, t, *J* 6.0 Hz), 2.5br (1 H, s), and 3.2 (4 H, s).

3-Ethylglutaric acid (8 g, 0.05 mol) and acetic anhydride (100 ml) were heated under reflux for 10 h. Excess of acetic anhydride and acetic acid were then removed by distillation. The residue was distilled *in vacuo* to give 3-ethylglutaric anhydride (5.2 g, 72%), b.p. 119—121 °C at 0.25 mmHg (lit.,[21] 158 °C at 13 mmHg); $\nu_{max.}$ (film) 1 725 and 1 825 cm^{-1}; δ($CDCl_3$) 1.0 (3 H, t, *J* 6.0 Hz), 1.2—1.7 (2 H, m), 1.9—2.3 (1 H, m), and 2.4—3.2 (4 H, m).

The above anhydride (14.2 g, 0.1 mol) was dissolved in dry tetrahydrofuran (70 ml) and added with stirring over 30 min at 0 °C to sodium borohydride (3.8 g, 0.1 mol) in dry tetrahydrofuran (30 ml). Stirring was continued at 20 °C for 3 h and then 6N-hydrochloric acid (100 ml) was added cautiously, followed by water (100 ml). The mixture was extracted with diethyl ether (4 × 100 ml) and the ether solution dried ($MgSO_4$), rotary evaporated, and distilled to give 3-*ethylvalerolactone* (8.3 g, 65%), b.p. 84 °C at 1.5 mmHg, $\nu_{max.}$ (film) 1 750 cm^{-1}; δ($CDCl_3$) 1.0 (3 H, t, *J* 6.0 Hz), 1.2—3.0 (7 H, m), and 4.0—4.6 (2 H, m) (Found: C, 64.9; H, 9.85. $C_7H_{12}O_2$ requires C, 65.6; H, 9.45%).

3-Ethylvalerolactone (3.9 g, 0.03 mol) in dry tetrahydrofuran (30 ml) was added dropwise to lithium aluminium hydride (1.1 g, 0.03 mol) in dry tetrahydrofuran (100 ml). The mixture was refluxed for 14 h. Water (6 ml), followed by 15% aqueous sodium hydroxide (2 ml), was then added cautiously to the cooled mixture. The filtered solution was dried ($MgSO_4$), rotary evaporated, and distilled. The 3-*ethylpentane*-1,5-*diol* (1c) (3.3 g, 82%) obtained had b.p. 115—116 °C at 1.25 mmHg; δ($CDCl_3$) 0.9 (3 H, t, *J* 6.0 Hz), 1.1—1.8 (7 H, m), and 3.3—4.2 (6 H, m) (Found: C, 63.6; H, 12.4. $C_7H_{16}O_2$ requires C, 63.6; H, 12.2%).

3-*Propylpentane*-1,5-*diol* (1d). The general procedure described above for (1c) was applied as outlined in the following sequence: diethyl n-propylmalonate [19] ⟶ 2-n-propylpropane-1,3-diol (72%), b.p. 128—130 °C at 1.5 mmHg ⟶ bismethanesulphonate (87%) ⟶ 3-n-propylglutaric acid (35%), m.p. 52—54 °C (lit.,[20] 52 °C); δ[$(CD)_3SO$] 0.7—1.1 (3 H, t, *J* 6.0 Hz), 1.1—1.6 (5 H, m), and 2.3 (4 H, s) ⟶ 3-n-propylglutaric anhydride (80%), b.p. 125—126 °C at 0.25 mmHg (lit.,[22] 180 °C at 20 mmHg); $\nu_{max.}$ (film) 1 725 and 1 825 cm^{-1}; δ($CDCl_3$) 1.0 (3 H, t, *J* 6.0 Hz), 1.2—1.7 (4 H, m), and 2.2—3.2 (5 H, m) (Found: C, 61.65; H, 7.6. $C_8H_{12}O_3$ requires C, 61.5; H, 7.75%) ⟶ 3-*n-propylvalerolactone* (82%), b.p. 90—91 °C at 1.5 mmHg; $\nu_{max.}$ (film) 1 750 cm^{-1}; δ($CDCl_3$) 1.0 (3 H, t, *J* 6.0 Hz), 1.2—2.9 (9 H, m), and 4.2—4.6 (2 H, m) (Found: C, 67.5; H, 9.85. $C_8H_{14}O_2$ requires C, 67.55; H, 9.85%) ⟶ 3-*n-propylpentane*-1,5-*diol* (1d) (80%), b.p. 101—103 °C at 0.1 mmHg; δ($CDCl_3$) 1.0 (3 H, t, *J* 6.5 Hz), 1.2—1.9 (9 H, m), and 3.7br (6 H, s) (Found: C, 65.75; H, 12.5. $C_8H_8O_2$ requires C, 65.7; H, 12.4%).

4-*Benzylpentane*-1,5-*diol* (1g). The general procedure for (1c) was used as follows: diethyl benzylmalonate [19] ⟶ 2-benzylpropane-1,3-diol (69%), b.p. 192—194 °C at 0.25 mmHg ⟶ bismethanesulphonate (86%) ⟶ 3-benzylglutaric acid (30%), m.p. 136—138° [from ethyl acetate–light petroleum (b.p. 60—80 °C)]; δ[$(CD_3)_2SO$] 2.1—3.0 (7 H, m) and 7.3 (5 H, s) ⟶ 3-benzylglutaric anhydride (81%), b.p. 128—130 °C at 0.25 mmHg, m.p. 83—84 °C; $\nu_{max.}$(CCl_4) 1 750 and 1 810 cm^{-1}; δ($CDCl_3$) 2.2—2.9 (7 H, m) and 7.0—7.6 (5 H, m) ⟶ 3-*benzylvalerolactone* (51%), b.p. 161—163 °C at 1 mmHg, m.p. 50—52 °C; $\nu_{max.}$ (film) 1 735 cm^{-1}; δ($CDCl_3$) 1.9—3.0 (7 H, m), 3.8—4.5 (2 H, m), and 7.0—7.5 (5 H, m) (Found: C, 75.7; H, 7.4. $C_{12}H_{14}O_2$ requires C, 75.75; H, 7.4%) ⟶ 3-*benzylpentane*-1,5-*diol* (1g) (90%), b.p. 158—159 °C at 0.05 mmHg; δ($CDCl_3$) 1.4—2.4 (3 H, m), 2.5—2.7 (2 H, m), 3.4—3.8 (8 H, m), and 7.2 (5 H, m) (Found: C, 74.05; H, 9.25. $C_{12}H_{18}O_2$ requires C, 74.2; H, 9.35%).

Rate Studies.—Oxidations. Kinetic runs were carried out at 25 °C in 0.05M-glycine–sodium hydroxide buffer of pH 9 in the presence of 5.0×10^{-4}M-NAD^+. The reactions were initiated by the addition of 10—100 μl of HLADH solution (1—2 mg ml^{-1} in 0.05M-Tris,HCl buffer of pH 7.4 assayed [4c] directly prior to use) to make a final volume of 3.00 ml in a 1 cm pathlength quartz cuvette. This was then shaken briefly to ensure mixing and placed in the constant temperature (25 °C) sample holder of a Cary 16 spectrophotometer equipped with a G-2500 recorder. The absorbance change at 340 mm was monitored. Each run was performed in duplicate and the initial rates at each substrate concentration were analysed in a Lineweaver–Burk manner by the least squares method. Correlation coefficients were $\geqslant 0.995$ in each case. The ϕ_0(app.) and ϕ_2(app.) parameters were calculated from the K_m(app.) and $V_{max.}$ values obtained as outlined in the Discussion section taking the molecular weight of HLADH as 84 000 [23] and the extinction coefficient of NADH as 6 560.[7b] The basic substrate concentration range used was 0.5×10^{-4}—1×10^{-2}M except when substrate activation or inhibition became a problem. The substrates for which this occurred, and the concentration restrictions thereby imposed, are noted in the Results section.

Reductions. The procedures were identical with that described above except that 0.1M-potassium phosphate buffer of pH 7.0, 1.75×10^{-4}M in NADH, was used.

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