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ON THE MECHANISM OF HYDROGEN TRANSFER BY NICOTINAMIDE COENZYMES AND SOME DEHYDROGENASES

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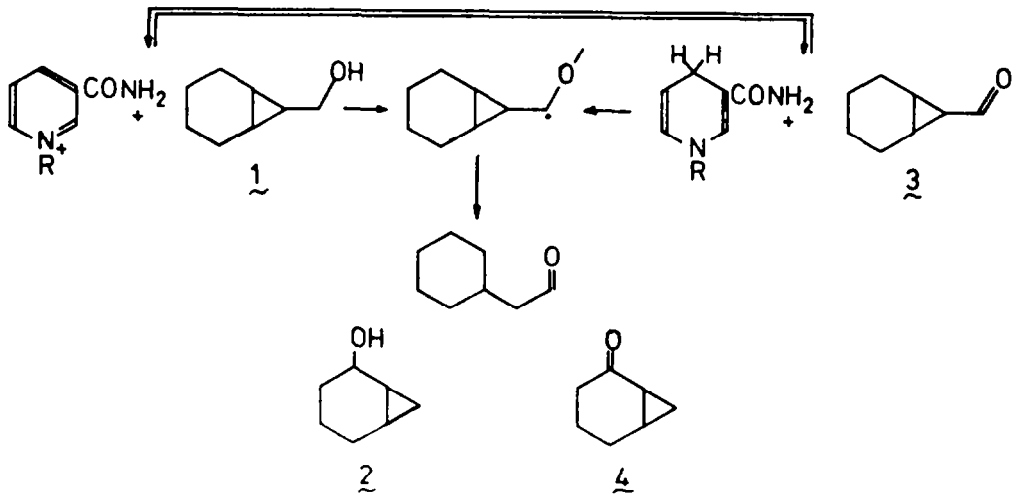
The course of the oxidation of 2,2-dimethylcyclopropylmethanol and 2,2,3,3-tetramethylcyclopropylmethanol by horse liver alcohol dehydrogenase and NAD^+ has been investigated. In neither case were ring opened products detected which, in agreement with previous observations, provides no evidence for the intermediacy of radicals in these reactions. The stability of several substituted cyclopropylalkyl radicals has been investigated by e.s.r. spectroscopy and the results are discussed with respect to cyclopropane-containing probes of the mechanism of oxidation of lactate dehydrogenase.

The mechanism by which hydrogen is transferred to and from dihydropyridines has been a subject of continuous debate over the last ten years. This debate has concerned many reactions, some catalysed by dehydrogenases,[1,2] but mostly model reactions using a wide variety of substrates, many distantly related to the carbonyl or imine substrates of enzymes. The pioneering study of these redox reactions carried out by Westheimer[3] suggested that hydrogen transfer from dihydropyridines was hydride-like. However 15 years later, Hamilton[4] argued that the possibility of proton and electron transfer should be seriously considered for these reactions and since then, many reactions have been investigated for signs of electron transfer mechanisms. In reactions of dihydropyridines with substrates that are predisposed to electron transfer such as nitro compounds, aromatic diazonium salts, and transition metal complexes, unequivocal evidence for radical pathways has been obtained.[5-12] Evidence has also been adduced from differences between kinetic and product isotope effects in dihydropyridine-mediated reductions of electrophilic compounds such as α,α,α-trihaloketones and N-methylacridinium salts that electron-transfer pathways were important.[13-22] However Bruice[23,24] has reinvestigated some of these reactions, especially systems relating to dihydropyridine reductions of flavins, and has concluded that hydride transfer is a better description of the mechanism. Bunting's studies of reductions of substituted isoquinolinium salts[25,26] have also favoured a hydride transfer. Reactions of simple aldehydes, ketones, and imines in which probes for radical intermediates have been used such as spin traps,[27] or cyclopropane-containing

substrates[1,2,28] have afforded no evidence for radical intermediates. It therefore seems that dihydropyridines are intrinsically flexible reducing agents capable of adapting to the chemical characteristics of the substrates and that a broad spectrum of possible mechanisms exists.[29]

Our chief concern in this field[1,2] along with Chung and Park[30] was to obtain the best possible evidence for the mechanism of hydrogen transfer by nicotinamide adenine dinucleotide coenzymes at the active site of horse liver alcohol dehydrogenase (HLADH) and lactate dehydrogenase (LDH). Kinetic experiments including studies of isotope and substituent effects have been carried out for enzyme-catalysed reactions[31-34] but the interpretation of such data in terms of a mechanism for hydrogen transfer is difficult because in enzyme-catalysed reactions, the hydrogen transfer step is usually only partially rate limiting. It was essential, therefore, to devise a probe for the mechanism of this reaction, for which (a) conclusions would not depend upon kinetic measurements alone, (b) sensitivity to short-lived intermediates was possible and (c) utilised substrates typical of those normally handled by the enzyme. With these criteria in mind, we selected the rapid ring opening reaction of cyclopropylmethyl radicals[35-37] as the basis for our first experiments[38,1] with HLADH. Similar considerations were published soon after by Chung and Park[30] and we also extended our experiments to lactate dehydrogenase.[2] The cyclopropylmethyl probe is applicable to probe the reaction mechanism of hydrogen transfer because ring opening of the unsubstituted radical occurs approximately 10^6 times faster than hydrogen transfer by HLADH and LDH.[39-42] Thus if a radical intermediate were involved, ring-opened products would be detected (Scheme 1).



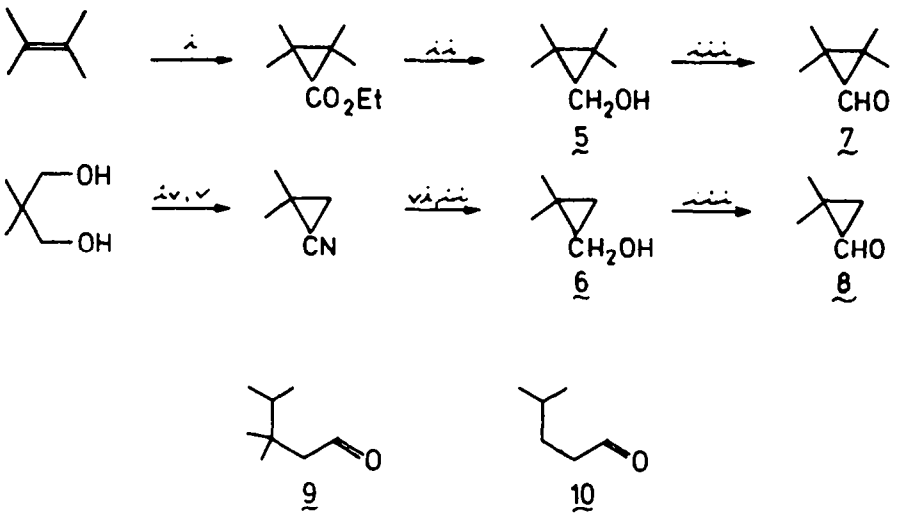
Scheme 1

In neither our experiments[1,2] nor Chung and Park's[30] was a ring-opened product derived from a radical intermediate detected. Thus no evidence for radical intermediates was obtained. However the limits of validity of conclusions drawn from such results depend upon two factors, namely the rate of ring opening of substituted cyclopropylmethyl radicals and the nature of the binding of the probe substrate at the enzyme's active site. With regard to the latter, it is conceivable that the substrate might be bound in a stereoelectronically unfavourable mode for ring opening.[43] HLADH has a capacious substrate-binding pocket and inspection of Jones' model[44] suggests that many conformations are available for the substrates that we studied (1-4) and hence stereoelectronic demands can be satisfied. A detailed discussion of the computer graphics modelling of substrates and inhibitors at the active site of HLADH will be published elsewhere. In summary we have found[45] that even for the largest inhibitors and probe substrates studied (1, 5) essentially free rotation is possible about the C_1-C_2 bond. Rotation about this bond controls the stereoelectronic relationship of the cyclopropane ring to the pro-R hydrogen that is removed during oxidation. This study also suggests an optimum geometry for inhibition of HLADH by cyclopropylmethanols.[46] Nevertheless, we are currently examining binding of substrates to HLADH by computer graphics techniques with regard to both the mechanism of hydrogen transfer and enzyme inhibition.[45]

Another approach to clarify the extent of the limitations of results obtained is to use other substrates that have different binding requirements and also undergo more rapid ring opening. In this paper, we present results bearing upon this discussion for both HLADH and LDH.

Results and Discussion

Hydrogen transfer by HLADH - From our previous studies,[1-47] the limit of detectability of a radical intermediate was of the order of 10^{-7} sec. since we found that trialkylsilylalkoxy cyclopropylmethyl radicals underwent ring opening approximately 5 times slower than the unsubstituted radicals at 298K. These results were obtained from variable temperature e.s.r. studies[47] in which typically both ring opened and unopened radicals could be observed together in the temperature range 130-170K. We have now examined 2,2,3,3-tetramethylcyclopropylmethanol (5) and 2,2-dimethylcyclopropylmethanol (6) with respect to their enzymic properties and rates of ring opening. The compounds were prepared as shown in Scheme 2.



Scheme 2 Reagents: i N_2CHCO_2Et/Cu; ii $LiAlH_4$; iii CrO_3/py; iv TsCl/py; v $KCN/HOCH_2CH_2OH$; vi aqNaOH

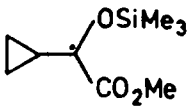
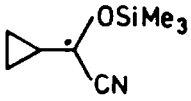
The trimethylsilylether of (5) was converted into the corresponding radical as before[47] by photolysis of a 1:1:5 mixture of the ether, di-t-butyl peroxide, and cyclopropane. In contrast to the ethers derived from cyclopropanemethanol itself, at 150K, no trace of the unopened radical was observed; only the ring opened radical $Me_2\dot{C}CMe_2CH{=}CHOSiMe_3$, was detected at temperatures down to 90K. The e.s.r. spectrum consisted of a broad septet [$\underline{a}$ (6H) = 22.9G]. This is equivalent to a rate of ring opening of at least 10^9 s^{-1} at 298K and hence the polymethyl-substituted cyclopropane methanols were suitable compounds to extend the limits of validity of our previous studies. Complete ring opening of the trimethylsilyl ether of (6) to give $Me_2\dot{C}CH_2CH = CHOSiMe_3$ [$\underline{a}$ (6H) = 23.2, $\underline{a}$ (2H) = 19.3, $\underline{a}$ (H) = 0.8] occurred at 120K, the lowest temperature used.

HLADH catalysed oxidations of (5) and (6) were carried out as before[1] following the conditions developed by Jones[51] and the products isolated by continuous extraction with ethyl acetate. The products were characterised by glc and 250 MHz ^1H n.m.r. For both probe molecules, the ^1H n.m.r. spectrum showed a new peak at δ9.3 corresponding to an aldehyde proton resonance. This peak was a clean doublet (J = 5.5 Hz) with both substrates, a result consistent only with an uncleaved cyclopropanecarboxaldehyde. Had ring opening occurred, the aldehyde proton would have appeared as a triplet. No alkene resonances were observed and the spectra were virtually completely assignable in terms of the probe alcohols (5), (6) and their corresponding aldehydes (7), (8). G.l.c. analysis reinforced this conclusion, the alcohols and aldehydes being the only significant products. No trace of the likely ring-opened products (9) or (10) was detected. Traces of the cyclopropanecarboxylic acids were detected : oxidation of the cyclopropanecarboxaldehydes

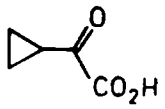
has been reported to occur very readily. Thus the use of more sensitive probes had failed to find evidence for radical intermediates in hydrogen transfer by HLADH.

Hydrogen transfer by LDH - Lactate dehydrogenase is a more difficult enzyme to study by means of modified substrates because of its obligatory requirement for the carboxylate to the site of oxidation/reduction. We found[2] that cyclopropaneglyoxylic acid (11) and cyclopropaneglycolic acid (12) were substrates for LDH and that clean oxidation or reduction reactions took place with no trace of ring opening. We also showed that the methyl ester of (11) underwent reduction with ring opening in the presence of tri-n-butyltin hydride at 80-120°. Reduction of the acid (11) by a model dihydropyridine was also found to occur with complete retention of the cyclopropane ring at 60°C. However, it was clearly important to determine, if possible, the rates of ring opening of the radical derived from (11) and (12) because the introduction of an α-carboxylate would be expected to enhance the stability of the radical greatly by the so-called capto-dative effect.[52] We decided to examine this possibility by studying the radical derived from the α-trimethylsilyloxy ester (13) (Table 1) : this radical was detectable with no evidence of ring opening up to 408K, a temperature above that at which ring opening occurred in our preparative reactions. A related radical derived from the α-trimethyloxynitrile (14) (Table 1) also showed no evidence of ring opening up to 305K. The remarkable stability of these radicals is regarded as evidence for capto-dative stabilisation of the radical centre.

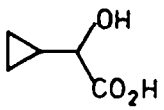
Table 1 E.s.r. Parameters of Substituted Cyclopropylmethyl Radicals[a]

	Temp.(K)	H.f.s.(G)
	150	1.48(4H), 1.11(2H), 0.74(3H)
	295	3.3(N), 2.2(3H), 1.1(2H)

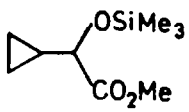
[a] In di-t-butyl peroxide



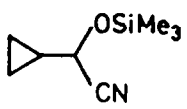
11



12



13



14

In the enzyme system it is likely that the acids (11 and 12) are effectively present as their carboxylate anions rather than the free acids, in which case the derived radicals might well possess less capto-dative stabilisation. We examined this by generating the radical from (11) with titanium(III)/hydrogen peroxide in a flow system over the pH range 2.1-9.2 at ambient temperature (293K). Once again there was no evidence of rearrangement.

In view of these results, our observation of lack of ring opening in the LDH-catalysed oxidation/reduction of (12) and (13) cannot be regarded as strong evidence against radical intermediates in this reaction. It could be argued that neither the esters nor the solvated carboxylates are good models for the state of a substate at the active site of LDH because the carboxylate of the substrate is intimately ion paired with a guanidinium cation from an arginine residue. Such a situation is difficult to reproduce in a system amenable to e.s.r. study. The best evidence available in this case remains the model reaction previously studied[2] which showed no evidence for radical intermediates. A recent study by Srinavasan and Fisher[57] on the reaction catalysed by glutamate dehydrogenase interpreted isotope effect data in terms of a hydride transfer mechanism. Thus the overwhelming body of evidence[1,2,23,24,27,28,30,57] argues that for biochemically significant substrates, nicotinamide coenzymes transfer hydrogen as hydride.

EXPERIMENTAL

Enzyme-catalysed oxidations were carried out as described previously[1] using HLADH (Sigma) without further purification. G.l.c. analysis of reaction products was carried out on a 5% FFAP on Chromosorb G column at 90°C.

Preparation of substrates and standard compounds

2,2,3,3,-Cyclopropylmethanol (5) This was synthesized by reaction of ethyl diazoacetate with 2,3-dimethylbut-2-ene[48] followed by reduction of the ethyl 2,2,3,3-tetramethyl-cyclopropanecarboxylate with lithium aluminium hydride,[3,49] and was obtained as a colourless liquid, b.p. 72°/12 mm (lit.[49] 72°/12 mm): δ_H ($CDCl_3$) 0.42 (1H, t, J, 8Hz), 1.02 (6H, s), 1.10 (6H, s), 1.50 (1H, s, exchanges on treatment with D_2O), 3.70 (2H, d, J = 8Hz).

2,2,3,3-Tetramethylcyclopropylmethyl trimethylsilyl ether To a stirred mixture of 2,2,3,3-tetramethylcyclopropylmethanol (1 g, 9 mmol) and triethylamine (0.9 g, 9 mmol) in dry pentane (50 ml), at 0°, was added over 10 min. chlorotrimethylsilane (1 g, 9 mmol). The mixture was allowed to warm up to room temperature, the triethylamine hydrochloride was filtered off under nitrogen, and the solvent distilled off. Kugelrohr distillation (90° at 20 mm) gave the trimethylsilyl ether (1.4 g, 80%) (Found: M^+ 200.1606. $C_{11}H_{24}OSi$

requires 200.1596); δ_H 0.10 (9H, s, $SiMe_3$), 0.35 (1H, t, J = 8Hz , $\underline{CH}CH_2$) 0.88 (6H, s, 2xMe), 0.99 (6H, s, 2xMe), 3.54 (2H, d, J = 8Hz, CH_2); δ_C -0.48 (q), 16.56 (q), 21.29 (s), 23.48 (q), 34.88 (d), 60.43 (t).

2,2-Dimethylcyclopropylmethanol(6) This was obtained in four steps[50] from 2,2-dimethylpropan-1,3-diol as a colourless liquid, b.p. 66°/12mm (lit.,[50] 95-96°/118mm): δ_H(250 MHz, $CDCl_3$) 0.134 (1H, dd, J,. 4.4 and 4.6 Hz), 0.474 (1H, dd, J 4.4 and 8.6 Hz), 0.918 (1H, dddd, J 4.4, 7.0, 8.4 and 8.6 Hz), 1.085 (3H, s), 1.123 (3H, s), 1.4 (1H, s, exchanges on treatment with D_2O),3.526 (1H, dd, J 8.4 and 11.5 Hz), 3.705 (1H, dd, J 7.0 and 11.5 Hz).

2,2-Dimethylcyclopropylmethyl trimethylsilyl ether This was prepared in the same way as 2,2,3,3-tetramethylcyclopropylmethyl trimethylsilyl ether. Kugelrohr distillation (110° at 80 mm) of the crude product gave the ether as a colourless oil (80%): δ_H (90 MHz, $CDCl_3$) 0.10-0.25 (1H, m), 0.15 (9H, s), 0.45 (1H, dd, J 4 and 8Hz), 0.55-0.70 (1H, m), 1.00 (6H, s), 3.50-3.65 (2H, m).

2,2,3,3-Tetramethylcyclopropanecarboxaldehyde(7) Oxidation of 2,2,3,3-tetramethylcyclopropylmethanol with chromium trioxide-pyridine in methylene chloride[53] gave the aldehyde, b.p. 82°/60mm (lit.,[49] 54°/13mm): δ_H (250 MHz $CDCl_3$) 1.14 (1H, dd, J, 4.6 and 7.9 Hz), 1.20 (3H, s,) 1.29 (3H, s), 1.36 (1H, t, J 4.9 Hz), 1.72 (1H, td, J 5.3 and 7.9 Hz), 9.34 (1H, d, J 5.3 Hz).

2,2-Dimethylcyclopropanecarboxaldehyde(8) Oxidation of 2,2-dimethylcyclopropylmethanol similarly gave the aldehyde,[54] b.p. 65°/80 mm: δ_H (250 MHz, $CDCl_3$) 1.14 (1H, dd, J, 4.6 and 7.9 Hz), 1.20 (3H, s), 1.29 (3H, s), 1.36 (1H, t, J 4.9 Hz), 1.72 (1H, td, J 5.3 and 7.9 Hz), 9.34 (1H, d, J 5.3 Hz).

Potassium 2-cyclopropyl-2-oxoacetate[55] (11) To a stirred mixture of cyclopropylmethyl ketone (75 g, 0.9 mol), sodium carbonate (1 g) and water (500 ml) at 50°, was added a solution of potassium permanganate (150 g) in water (4.5ℓ) dropwise over 10 h. When the addition was complete, methanol (500 ml) was added to the mixture and then filtered off. The solvent was removed from the filtrate by freeze-drying giving the potassium salt (84 g, 61%), m.p. 255-259 decomp. (lit.,[55] 258 decomp.). A small portion of the salt was crystallized from acetone giving white crystals, m.p. 257-259° decomp.: δ_H (D_2O) 1.1-1.3 (4H, m), 2.2-2.5 (1H, m).

Methyl 2-cyclopropyl-2-oxoacetate A solution of the above potassium salt (50 g, 0.33 mol) in water (100 ml) was vigorously stirred with a solution of dimethyl sulphate (50 g, 0.39 mol), benzyl trimethylammonium bromide (9 g, 3.9 mmol) in dichloromethane at room temperature for 48 h. The aqueous phase was separated, extracted with dichloromethane (2 x 25 ml). The combined organic layers were washed with water, and dried (Na_2SO_4). The

residue, after removal of the solvent, was distilled to give the ester, b.p. 97–98°/20 mm (lit.,[55] 85–90°/12 mm): δ_H ($CDCl_3$) 1.1–1.3 (4H, m), 2.6–2.9 (1H, m), 3.9 (3H, s).

Methyl 2-cyclopropyl-2-hydroxyacetate A mixture of the above ester (9 g, 70 mmol) and sodium cyanoborohydride (3.5 g, 55 mmol) in dry methanol (250 ml) containing a trace of bromocresol green indicator. A solution of hydrogen chloride in dry methanol was added dropwise to maintain the solution at pH 4. After 8 h, the methanol was distilled off and the residue acidified with dil. hydrochloric acid. The mixture was extracted with ether, washed with saturated brine, saturated sodium hydrogen carbonate solution, and dried ($MgSO_4$). After removal of the solvent, the residue was distilled to give the hydroxy ester (5.5 g, 60%), b.p. 80°/20 mm: δ_H ($CDCl_3$) 0.4–0.6 (4H, m), 0.95–1.30 (1H, m), 3.00 (1H, s, exchanges with D_2O), 3.81 (1H, d, *J* 8Hz), 3.85 (3H, s): δ_C (CD_3OD) 1.82 (t), 14.80 (d), 52.54 (q), 72.93 (d), 175.70 (s).

Methyl 2-cyclopropyl-2-trimethylsilyloxyacetate (13) Chlorotrimethylsilane (1.7 g, 15 mmol) was added slowly to a stirred mixture of the above hydroxy-ester (1.8 g, 14 mmol) and triethylamine (16 mmol) in anhydrous ether (50 ml) at 0°. After stirring for 2 h, the amine hydrochloride was filtered off and the solvent distilled off. Kugelrohr distillation (140° at 20 mm) gave the trimethylsilyloxyacetate (2.5 g, 86%), (Found: M^+ 201.0920. $C_9H_{17}O_3Si$ requires 201.0947): δ_H ($CDCl_3$) 0.15 (9H, s), 0.4–0.6 (4H, m), 0.9–1.3 (1H, m), 3.75 (1H, d, *J* 8Hz); δ_C ($CDCl_3$) −0.61 (q), 1.33 (t), 2.18 (t), 14.62 (d), 51.33 (q), 74.26 (d), 173.45 (s).

2-Cyclopropyl-2-trimethylsilyloxy acetonitrile (14) Dry potassium cyanide (65 g, 1 mmol) dried in vacuo 100°/0.1 mm Hg) and 18-crown-6 ether in dry methanol (15 ml) were stirred together for 15 min. The solvent was evaporated off and the residue dried on a vacuum line for 10 min (0.01 mm). To a stirred mixture the residual complex and cyclopropanecarboxaldehyde (2.1 g, 30 mmol) was added over 30 min trimethylsilyl cyanide (3.1 g, 31 mmol). The mixture was stirred overnight and then distilled three times using a Kugelrohr apparatus (85° at 15 mm) to give 2-cyclopropyl-2-trimethylsilyloxy acetonitrile (4 g, 80%) (Found: C, 56.5; H, 9.05; N, 8.25%. $C_8H_{15}NOSi$ requires C, 56.8; H, 8.9; N, 8.3%): δ_H ($CDCl_3$) 0.20 (9H, s), 0.45–0.70 (4H, m), 1.30–1.45 (1H, m), 4.12 (2H, d, *J* 7 Hz); δ_C ($CDCl_3$) 0.30 (q), 2.43 (t), 3.88 (t), 16.50 (d), 65.10 (d), 119.82 (s).

E.s.r. experiments A mixture of the cyclopropyl compound, di-t-butyl peroxide, and cyclopropane in the ratio 1:1:15 by volume, or a 1:5 mixture of the cyclopropyl compound in di-t-butyl peroxide was degassed and sealed in spectrosil tubes and photolysed with light from a 500W high-pressure mercury arc in the cavity of a Bruker ER 200D spectrometer.

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