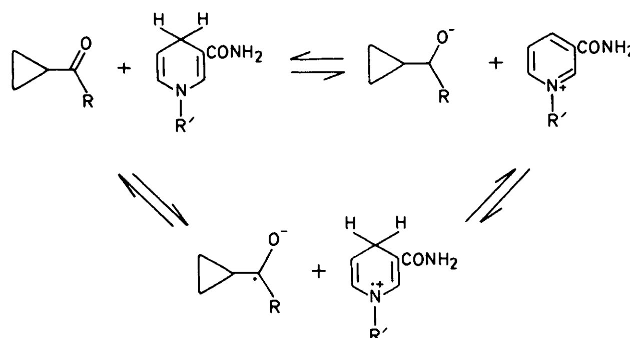
On The Mechanism of Hydrogen Transfer by Nicotinamide Coenzymes and Alcohol Dehydrogenase

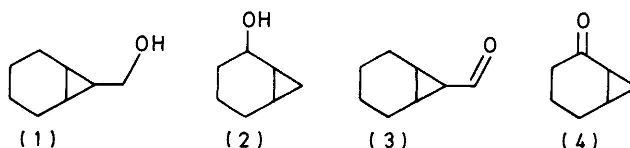
Iain MacInnes, Derek C. Nonhebel, Stefan T. Orszulik, and Colin J. Suckling *
Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL

The oxidation of *exo*-bicyclo[4.1.0]heptan-7-ylmethanol and of bicyclo[4.1.0]heptan-2-ol and the reduction of the corresponding carbonyl compounds by horse liver alcohol dehydrogenase (E.C.1.1.1.1) occur without cleavage of the three-membered ring. Reactions of these substrates with hydride donating and abstracting reagents also proceed with retention of the small ring whereas radical reactions lead to cleavage of the cyclopropane ring. These results, together with kinetic studies on the rates of ring opening of cyclopropylmethyl silyl ethers by e.s.r. spectroscopy suggest that the enzyme-catalysed reaction takes place through transfer of hydrogen as hydride.

Westheimer's classic model study of reduction reactions of 1,4-dihydropyridines (NADH models) was for many years accepted as indicating a favoured hydride-like mechanism for these reactions.[1] Fifteen years later, in a thought provoking review, Hamilton argued that proton transfer was by far the most probable mechanism for biological hydrogen transfer [2] and these ideas stimulated a resurgence of interest in 1,4-dihydropyridine chemistry especially with regard to the possible occurrence of one electron transfer reactions leading to radical intermediates. Model reactions were found in which one electron transfer reactions were demonstrated.[3-6] However, in such cases, the substrates were often predisposed to one electron reduction and quite unlike any of the natural enzyme substrates. Isotope effect studies of model reductions of reactive carbonyl compounds and *N*-methylacridinium salts were also advanced as reactions in which intermediates from one electron transfer were possible.[7-16] Recent investigations by Bruice [17] have shed doubt upon the validity of some of the data and an elegant study of hydrogen transfer between 1,4-dihydropyridines and isoquinolinium salts by Bunting [18,19] has indicated a substantial negative charge upon the hydrogen atom in the transition state. Despite this evidence and a negative model spin trapping study,[20] there have been persistent assertions, principally from Japanese workers [9-12] that these hydrogen transfer reactions involve radical-like intermediates. All of the model studies cited above, whilst providing important background information, do not bear directly upon the mechanism of the enzyme-catalysed reactions. It is difficult to obtain easily interpretable kinetic data pertaining to the hydrogen transfer step in the enzyme-catalysed reaction since it is only partially rate limiting. For this reason, data from substituent effects with aromatic substrates were not easy to interpret in terms of a mechanism for hydrogen transfer, although hydride mechanisms have usually been preferred.[21-24] There was clearly a need for a mechanistic probe that would focus attention upon the redox step and that would minimise the need to extrapolate a mechanism from the behaviour of model compounds. Bearing in mind the catholic substrate requirements of horse liver alcohol dehydrogenase, we realised that a potential probe of the mechanism of hydrogen transfer would be a cyclopropylmethyl derivative (Scheme 1). Ingold and others have demonstrated that cyclopropylmethyl radicals undergo remarkably rapid ring opening to the corresponding homoallyl radical; [25-27] the rate constants for these reactions are of the order of 10^8 s^{-1}. In contrast, the rate of ring opening of derivatives of cyclopropylmethanols in solvolysis reactions has been found to be some 10^{11} times slower.[28,29] Rates of enzyme-catalysed hydrogen transfer have been estimated as being at most close to 100 s^{-1}.[30-33] It therefore seemed to us



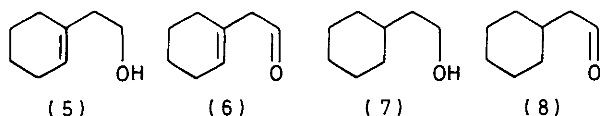
Scheme 1. R = H or alkyl



probable that if the enzyme-catalysed redox reaction proceeded through a radical intermediate, ring opening would occur but that a hydride transfer mechanism would leave the cyclopropane ring intact. In this way, it would be possible to probe the mechanism of hydrogen transfer using a reporter molecule that was itself an enzyme substrate.[34] Two cyclopropylmethanols were chosen for study, bicyclo[4.1.0]-heptan-7-ylmethanol (1) and bicyclo[4.1.0]heptan-2-ol (2); these compounds were expected to bind well to horse liver alcohol dehydrogenase.[35,36] The syntheses of these molecules and the corresponding carbonyl compounds (3) and (4) have been reported in the preceding paper. In this paper, we describe the results of preparative enzyme-catalysed redox reactions of these substrates and assess the observations as evidence for the nature of hydrogen transfer catalysed by the enzyme.

Results and Discussion

Preparative Enzymic Experiments.—As was shown in the preceding paper, the probe molecules are substrates for horse liver alcohol dehydrogenase with kinetic parameters similar to those of related compounds not containing cyclopropyl groups. Each of the alcohols (1) and (2) was incubated with alcohol dehydrogenase under the NAD cofactor recycling conditions developed by Jones.[35] After 3 days' incubation,



the products of reaction were isolated by continuous extraction with ethyl acetate and were characterised by g.l.c. on two columns and by ^{1}H n.m.r. spectroscopy in comparison with authentic reference compounds. We found that for the methanol (1), the products consisted of a mixture of unchanged starting material and the aldehyde (3) in a 7 : 3 ratio at 85% recovery, together with a small quantity (10%) of 2-vinylcyclohexanol. As explained in the preceding paper, this last product was probably derived by enzyme-catalysed solvolysis since it was also obtained in reactions in the presence of the reducing coenzyme, NADH, in which hydrogen transfer was impossible. Under the same conditions, the alcohol (2) was oxidised quantitatively to the corresponding ketone (4). The reduction reactions of the aldehyde (3) and the ketone (4) were also investigated, recycling NADH with sodium dithionite.[35] From the aldehyde (2) we obtained a 30% yield of the alcohol (1) together with unchanged aldehyde; however the ketone (4) failed to undergo reduction under these conditions. The evidence of these reactions shows that no ring opening occurred during hydrogen transfer. The likely products (5)—(8) of ring opening of the alcohol (1) through radical intermediates were synthesised, but none were detected.

Chemical Properties of the Probes.—Before drawing mechanistic conclusions from these observations, we wished to confirm that the probe molecules underwent radical and anionic hydrogen transfer in the manner expected from precedent. The reduction of bicyclo[4.1.0]heptane-7-carboxylic acid by lithium aluminium hydride and of the ketone (4) by sodium borohydride proceeded as expected with retention of the small ring. Oxidation of the trimethylsilyl ethers of (1) and (2) by trityl tetrafluoroborate,[37] a hydride abstracting reagent, also took place without ring opening. However reduction of the aldehyde (3) with tri-n-butyltin hydride (2 equiv.) in the presence of azobisisobutyronitrile as initiator afforded an 86% yield of cyclohexylethanol.[38,39] Davies [40] has already demonstrated ring opening of the alkoxystannyl radical derived from (4) using e.s.r. spectroscopy. It thus appears that the compounds chosen as probes for the mechanism of hydrogen transfer catalysed by alcohol dehydrogenase fit the pattern of behaviour expected from precedent. Radical intermediates are therefore improbable in the enzyme-catalysed reaction.

To define the limits of validity of the conclusion drawn from the preparative reactions described above, we wished to determine quantitatively the effect of an alkoxy substituent upon the rate of ring opening of the substituted cyclopropylmethyl radical. Variable temperature e.s.r. studies of the ring opening of a series of primary and secondary cyclopropylalkyl trialkylsilyl ethers were therefore carried out in collaboration with Walton.[41] The results are reproduced in the Table and show clearly that rapid ring opening also occurs with these substituted cyclopropylalkyl radicals. If radical intermediates are formed in the enzyme-catalysed reaction, they must have a lifetime of less than 10^{-8} s.

Since the compounds that we have used as probes are typical substrates for this enzyme, mechanistic conclusions drawn from these results involve a minimum of extrapolation. Inspection of Jones' model of the active site of HLADH [42] with the alcohol (1) bound suggests that there are several conformations available for this substrate. If this is so, the prevention of ring opening by a stereoelectronically disfavoured conformation of (1), as pointed out by Chung and Park,[43] may not be a problem. We have found no evidence for radical intermediates in redox reactions catalysed by horse liver alcohol dehydrogenase. Indeed parallel studies on lactate dehydrogenase [44] led to similar conclusions. Such evidence as exists for electron transfer reactions in 1,4-dihydropyridine chemistry is confined to reductions of one electron oxidants. We therefore believe that redox reactions of nicotinamide coenzymes with substrates typical of enzymes should be regarded as hydride-like.

Table. Rates of ring opening of cyclopropylmethyl radicals [41]

Radical	k (298 K)/s^{-1}
cyclopropyl–ĊH–OSiMe$_3$	1.7×10^{7}
cyclopropyl–ĊH–OSiBu^{t}Me$_2$	3.5×10^{7}
cyclopropyl–Ċ(Me)–OSiMe$_3$	2.4×10^{7}

Experimental

exo-Bicyclo[4.1.0]heptan-7-ylmethanol (1), *exo*-bicyclo[4.1.0]-heptane-7-carbaldehyde (3), bicyclo[4.1.0]heptan-2-ol (2), and bicyclo[4.1.0]heptan-2-one (4) were obtained by the methods described in the preceding paper. *2-Cyclohex-*1-*enylethanol* (5) was prepared from 1-chlorocyclohexene by lithiation [45] and reaction with ethylene oxide. The product had b.p. 102 °C at 20 Torr, ^{1}H n.m.r. ($CDCl_3$, 60 MHz), 1.55 (4 H, m), 1.90 (4 H, m), 2.12 (2 H, t, J 7 Hz), 3.55 (2 H, t, J 7 Hz), and 5.40 (1 H, bds) (Found: C, 75.8; H, 11.3. $C_8H_{14}O$ requires C, 76.14; H, 11.18%). Cyclohex-1-enylacetaldehyde[46] (6) was prepared by oxidation of the above alcohol with CrO_3–pyridine.[47] 2-Cyclohexylethanol [48] (7) and cyclohexylacetaldehyde [48] (8) were prepared by catalytic hydrogenation of the above compounds.

Preparative enzyme-catalysed redox reactions were carried out as described by Jones [49] during 3 days adding further portions of enzyme each day. The products were then extracted continuously with ethyl acetate and analysed by ^{1}H n.m.r. spectroscopy and g.l.c. on two columns (A, 5% FFAP on Chromosorb G and B, 5% Apiezon on Chromosorb G). None of the ring-opened compounds (5)—(8) was detected by g.l.c. or n.m.r. and the identity of the products (1)—(4) was confirmed by coinjection of authentic samples. Typical retention times were: on A at 120 °C (1) 18.0, (3) 11.0, (5) 16.0, (6) 5.5, (7) 16.5, (8) 5.5 min; and on B at 140 °C (1) 6.4, (3) 7.0, (5) 4.4, (6) 3.0, (7) 4.4, (8) 2.3 min at a flow rate of *ca.* 25 ml min^{-1}.

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