2-Cyclopropaneglyoxylic Acid: a Probe for the Mechanism of Hydrogen Transfer by Lactate Dehydrogenase and by *N*-Benzyl-1,4-dihydronicotinamide

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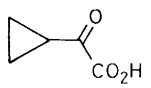
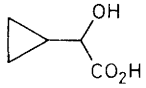
Reduction of cyclopropaneglyoxylic acid by lactate dehydrogenase and by model systems for both lactate and alcohol dehydrogenases occurs without cleavage of the three-membered ring implying that radical intermediates are improbable in these reactions.

In a previous communication,[1] we described how cyclopropylmethyl analogues of enzyme substrates can be used as mechanistic probes of hydrogen transfer by nicotinamide coenzymes and we showed that the three-membered ring remained intact in reactions catalysed by horse liver alcohol dehydrogenase; the results argued against the intermediacy of radicals. This enzyme contains zinc cations at its active site and since it has been argued that metal cations favour one-electron transfer,[2] we wished to apply the same probe technique to a dehydrogenase that does not employ a metal ion. Lactate dehydrogenase, which uses imidazolium as its acid–base catalyst, was chosen for study.

The appropriate substrate, cyclopropaneglyoxylic acid (**1**) was readily available from cyclopropyl methyl ketone.[3] This keto-acid binds to lactate dehydrogenase (Böhringer, pig heart) with affinity typical of a substrate [K_m (**1**) = 7.2×10^{-3} M at pH 7.4]. The potassium salt of (**1**) (0.015 M) was reduced preparatively by lactate dehydrogenase (3 units added daily in equal portions), NAD^+ (2.3×10^{-3} M) in 0.07 M phosphate buffer, pH 7.4 using horse liver alcohol dehydrogenase and an excess of ethanol to recycle the coenzyme. After reaction for 3 days, the products were extracted with ethyl acetate. ^{1}H N.m.r. spectroscopy and h.p.l.c. (anion exchange column at pH 3) showed that reduction had taken place quantitatively to yield cyclopropaneglycolic acid (**2**). The reverse reaction, enzyme-catalysed oxidation, was carried out under conditions similar to those for related reactions with alcohol dehydrogenase.[1] Thus cyclopropaneglycolic acid (0.05 M) FMN (0.08 M), and NAD^+ (4.7×10^{-3} M) were incubated at 37 °C for 3 days with lactate dehydrogenase (3 units added daily in equal proportions) in 0.1 M phosphate buffer, pH 9. A mixture of carboxylic acids was extracted as before and h.p.l.c. (reverse phase ion pair chromatography) showed that the mixture comprised cyclopropaneglyoxylic acid (**1**) (61%) and unchanged starting material (**2**) (39%). No ring-opened products were detected.



(1)



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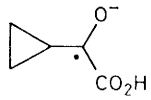

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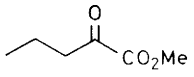

(4)

Before mechanistic inferences can be made from these results, it must be shown that the putative radical intermediate (**3**) does indeed undergo ring opening. This was demonstrated by treatment of the methyl ester of (**1**), conveniently prepared from the potassium salt using dimethyl sulphate under phase transfer catalytic conditions, with tri-n-butyltin hydride[4] in the presence of azoisobutyronitrile at 80—120 °C. The product of this reaction was methyl 2-oxopentanoate (**4**) as shown by g.l.c. (5% FFAP on Chromosorb G at 120 °C). This result indicates that the chosen substrates conform to the normal pattern of behaviour of substituted cyclopropylmethyl radicals. In agreement with alcohol dehydrogenase, therefore, it is improbable that hydrogen transfer from nicotinamide coenzymes occurs *via* radical intermediates.

Reports of radical intermediates in dihydropyridine chemistry chiefly concern model reactions[2,5] in particular with substrates very different from the natural carbonyl compounds that enzymes require.[6] Ohno[7] has shown that α-dicarbonyl compounds are readily reduced by model nicotinamide cofactors such as *N*-benzyl-1,4-dihydronicotinamide.

Cyclopropaneglyoxylic acid also provides an opportunity to apply the mechanistic probe to a model reaction. Thus treatment of a suspension of the keto-acid salt (**1**) (0.015 M) and magnesium perchlorate (0.015 M) in dry acetonitrile at 60 °C with *N*-benzyl-1,4-dihydronicotinamide (0.015 M) for 18 h resulted in quantitative conversion into the corresponding hydroxy-acid (**2**) in which the ring was intact as shown by ^{1}H n.m.r. spectroscopy and h.p.l.c. The reaction can be considered a model for alcohol dehydrogenase and our results corroborate the negative spin trapping study of Prince.[8]

A model system for lactate dehydrogenase is more difficult to construct because of the susceptibility of the dihydropyridine to protonation at C-5. However we found that in the presence of imidazolium chloride (0.03 M), the keto-acid (**1**) (0.015 M) was partially reduced in acetonitrile at 60 °C by *N*-benzyl-1,4-dihydronicotinamide (0.015 M) once again without cleavage of the three-membered ring. The reaction was approximately 10% complete by ^{1}H n.m.r. spectroscopy which also indicated that the major reactions involved reduction of the iminium group of the imidazolium cation and addition of the latter to C-5 of the dihydropyridine ring. H.p.l.c. analysis of the products failed to detect any ring-opened carboxylic acids.

The results presented here, and previously,[1] fail to implicate radical intermediates in any of the enzymic or model reactions studied. We therefore consider that hydrogen transfer between 1,4-dihydropyridines or pyridinium salts and substrates typical of those commonly transformed by enzymes should be regarded as hydride-like.

We thank the S.E.R.C. for financial support (S. T. O.). This work was carried out during the tenure of the Royal Society Smith and Nephew Senior Research Fellowship (C. J. S.).

Received, 2nd August 1982; Com. 911

References

1 I. MacInnes, D. C. Nonhebel, S. T. Orszulik, and C. J. Suckling, *J. Chem. Soc., Chem. Commun.*, 1982, 121.
2 A. Ohno, H. Yamamoto, and S. Oka, *J. Am. Chem. Soc.*, 1981, **103**, 2041; A. Ohno, T. Shio, H. Yamamoto, and S. Oka, *ibid.*, 2045.
3 I. Basnak and J. Farkas, *Coll. Czech. Chem. Commun.*, 1975, **40**, 1038.
4 D. C. Nonhebel, C. J. Suckling, and J. C. Walton, *Tetrahedron Lett.*, in the press.
5 A. Ohno, H. Yamamoto, and S. Oka, *Bull. Chem. Soc. Jpn.*, 1981, **54**, 3489; A. Ohno and N. Kito, *Chem. Lett.*, 1972, 369; Y. Ohnishi and A. Ohno, *ibid.*, 1976, 697; T. Endo and M. Okawara, *ibid.*, 1977, 1487.
6 R. J. Kill and D. A. Widdowson, *J. Chem. Soc., Chem. Commun.*, 1976, 755; T. Okamoto, A. Ohno, and S. Oka, *ibid.*, 1977, 181; S. Shinkai, T. Tsuno, and O. Manabe, *ibid.*, 1982, 592; N. Ono, R. Tamura, and A. Kaji, *J. Am. Chem. Soc.*, 1980, **102**, 2851.
7 Y. Ohnishi, M. Kagami, and A. Ohno, *Tetrahedron Lett.*, 1975, 2437; A. Ohno, H. Yamamato, T. Kimura, and S. Oka, *ibid.*, 1976, 4585.
8 R. A. Hood, R. H. Prince, and K. H. Rubinson, *J. Chem. Soc., Chem. Commun.*, 1978, 300.