Reactive Intermediates in Enzyme-catalysed Reactions*

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1 Introduction

It has often been remarked that enzymes catalyse reactions in a subtle manner, making full use of the inherent reactivity of their substrates. Although the reactions mediated by enzymes are sometimes surprising at first sight, a closer study reveals that the mechanisms involved are closely akin to those found in solution reactions. Training in organic chemistry emphasizes the mechanistic relationships between solution reactions and a major part of such generalizations centres upon reactive intermediates. The factors that influence the reactivities of carbanions, radicals, carbocations, and carbenes are familiar fare. Similarly the relationship between the stability of a reactive intermediate and the rate of a reaction is also familiar; since such intermediates are high energy species, they will usually be closer in character to the transition state for the reaction than either the starting materials or the products. Hence, if the intermediate can be stabilized in some way, a lowering in energy of the transition state will probably result also, and the reaction will be accelerated.

In the last ten years, significant advances have been made in our understanding of the basis of enzymic catalysis. The essence of catalysis by enzymes is to have the right reactants together at the right time and in the right place. This summarizes the entropic component of enzymic catalysis, that is enzyme-catalysed reactions are essentially made intramolecular by the ability of the enzyme to bind reactants at its active site. Several recent reviews deal with this topic.[1-3] However, once reaction is in progress at the active site, an enzyme still has to deal with the formation and utilization of reactive intermediates. It has been known for many years how enzymes deal with the problem of stabilizing carbanions but only recently have the enzyme chemistries of radicals, carbocations, and carbenes been placed upon a firm experimental basis.

*This review is based upon one section of an Irvine Review Lecture given at the University of St. Andrews, November, 1982, and it is the author's hope that the presentation of the topic through the familiar frame of the reactive intermediate will be instructive to the general reader and will also offer the practising organic chemist a new perspective on the important topic of enzymic catalysis. Many of the subjects described are considered in more detail in 'Enzyme Chemistry, Impact and Applications', ed. C. J. Suckling, Chapman and Hall, London, 1984 and references to appropriate chapters will be cited in this review.

[1] R. Kluger in 'Enzyme Chemistry, Impact and Applications', ed. C. J. Suckling, Chapman and Hall, London, 1984, chapter 2.
[2] A. J. Kirby in 'Comprehensive Organic Chemistry', vol. 5, ed. E. Haslam, Pergamon, Oxford, 1979 p. 389.
[3] W. N. Lipscomb, *Acc. Chem. Res.*, 1982, **15**, 322.

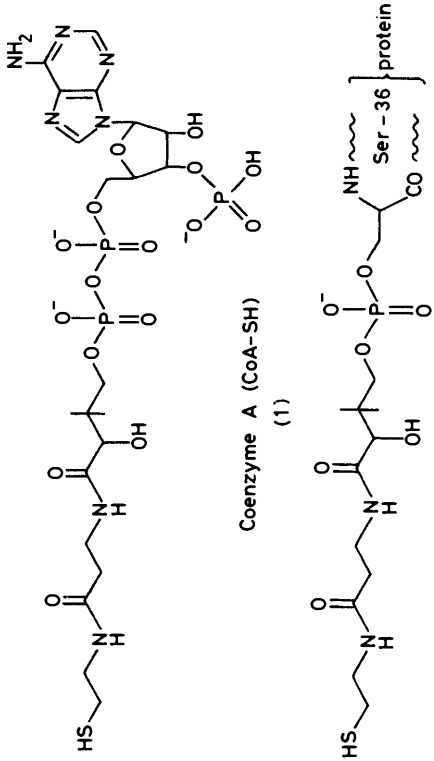


Figure 1 *Coenzyme A and Acyl Carrier Protein*

This article discusses some modern aspects of the chemistry of these reactive intermediates at enzyme active-sites and draws parallels, where appropriate, with synthetic chemistry or with topics of current mechanistic significance.

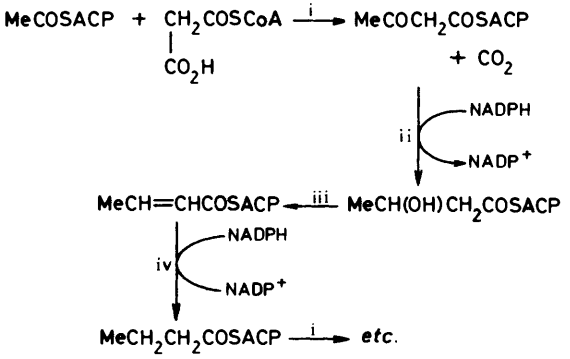
2 Carbanions

Condensation reactions involving carbanions form one of the largest classes of synthetic methods for constructing the carbon skeleton of an organic compound.[4,5] This is as true of biosynthetic reactions as of laboratory synthesis. The chief contrast between the two fields is that biological reactions substitute for the controlling techniques of low temperatures and sterically hindered bases typical of modern synthetic organic chemistry the ordered precision of the enzyme's active site. Although an enzyme can exert considerable control upon the course of possible reactions of its substrate, it still has to overcome the problem of generating carbanions. Strong bases are, of course, not available from the side-chains of protein amino-acids and consequently, when carbanions are required, enzymes must ensure that the anions are stabilized to achieve catalysis. Three coenzymes are especially important in this regard, coenzyme A (CoA) and the related acyl carrier protein (ACP), thiamine pyrophosphate (TPP) and pyridoxal phosphate (PLP). The salient features of these coenzymes have been understood for some time and have been extensively discussed.[6–8]

A. Coenzyme A and Acyl Carrier Protein.—The familiar Claisen ester condensation is one of the fundamental base-catalysed synthetic reactions of organic chemistry. Formally, there are close parallels between the carbon–carbon bond forming reactions in the biosynthesis of fatty acids and polyketides and this condensation. However, the enzyme reactions employ thiol esters in place of alcohol esters and the thiols are provided by CoA (1) or ACP (2) (Figure 1). With respect to their oxygen-containing analogues, thiol esters are more readily attacked at the carbonyl group and the α-protons are more acidic. In an alcohol ester, the non-bonded electrons of the alcohol oxygen atom can be considered to be delocalized into the carbonyl group. In the sulphur analogue, however, delocalization of the more diffuse 3*p* non-bonded electrons is less effective and consequently the carbonyl group of a thiol ester has reactivity more typical of a ketone than an ester. In quantitative terms the difference can be seen in the pK_a values of related β-dicarbonyl compounds: a typical β-ketoester 10.5; a β-diketone 8.2—8.9, and a β-ketothiolester 8.5.[9] It was therefore tempting to rationalize the

[4] R. K. Mackie and D. M. Smith, 'Guidebook to Organic Synthesis', Longmans, London, 1982, pp. 83—93.
[5] R. O. C. Norman, 'Principles of Organic Synthesis', 2nd edn., Chapman and Hall, London, 1978, p. 225.
[6] H. C. S. Wood in 'Comprehensive Organic Chemistry', vol. 5, ed. E. Haslam, Pergamon, Oxford, 1979, p. 489.
[7] H. Dugas and C. Penney, 'Bioorganic Chemistry', Springer Verlag, New York, 1981.
[8] K. E. Suckling and C. J. Suckling, 'Biological Chemistry', Cambridge University Press, Cambridge, 1980.
[9] T. C. Bruice and S. J. Benkovic, 'Bioorganic Mechanisms', vol. 1, Benjamin, New York, 1966, p. 297.

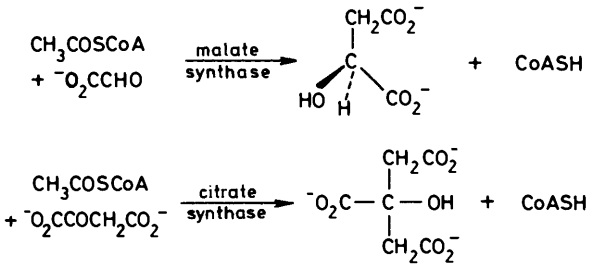
biosynthesis of fatty acids as involving carbanions in the chain-extending reaction (Scheme 1) and it came as a surprise when Lynen, who had played a major role



Scheme 1

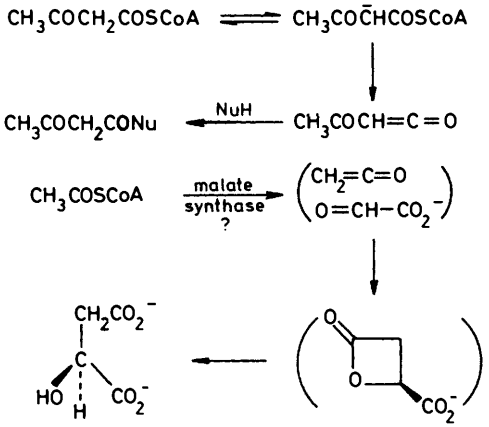
in elucidating the significance and general chemical characteristics of thiol esters, showed that carbanions were not intermediates in this reaction.[10] He was able to isolate the required enzyme and to prepare malonyl-ACP, the chain-extending unit. Consistent with the ease of ionization of the protons α to the thiol ester, it was found that these protons rapidly exchanged with deuterium in D_2O. If a carbanion is formed in the enzyme-catalysed reaction, it would be expected that an isotope effect on the reaction rate would be observed when the deuteriated substrate was used. None was found. A further piece of evidence against the formation of carbanions was that no tritium was incorporated into the product when the reaction was run in tritiated water. As the equation shows (Scheme 1, reaction 1), this reaction also involves a decarboxylation and loss of carbon dioxide can provide the nucleophilic reactivity required for condensation. Lynen argued that the isotope experiments showed that decarboxylation and condensation are concerted events in the enzyme-catalysed reaction. It is often easy to draw formal analogies between the mechanisms of simple chemical reactions and biochemical ones but, as in this case, there can be substantial differences. There are, however, coenzyme A mediated reactions in which carbanions do appear to be involved. The stereochemistry of dehydration of hydroxyacyl-CoAs, analogous to step 3 in the biosynthesis of fatty acids (Scheme 1) suggests that an *E*1CB-type mechanism is operating[11,12] and significant primary deuterium isotope effects have been measured for the reactions catalysed by malate and citrate synthases (Scheme 2).[13–15]

[10] K.-I. Arnstadt, G. Schindbleck, and F. Lynen, *Eur. J. Biochem.*, 1975, **55**, 561.
[11] P. Willadsen and H. Eggerer, *Eur. J. Biochem.*, 1975, **54**, 247.
[12] B. Sedgwick, C. Bacquet, and S. J. French, *J. Chem. Soc., Chem. Commun.*, 1978, 193.
[13] J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Nature*, 1969, **221**, 1213.
[14] J. Luthy, J. Retey, and D. Arigoni, *Nature*, 1969, **221**, 1213.
[15] J. Retey, J. Seibl, D. Arigoni, J. W. Cornforth, G. Ryback, W. P. Zeylemaker, and C. Veeger, *Eur. J. Biochem.*, 1970, **14**, 232.



Scheme 2

Until very recently, this pattern of behaviour was generally accepted but Douglas has awakened some possibilities that have lain dormant for many years.[16] He has shown that some thiolesters can undergo acyl transfer reactions *via* ketene intermediates (Scheme 3) and suggests that electrocyclic addition of a ketene to an



Scheme 3

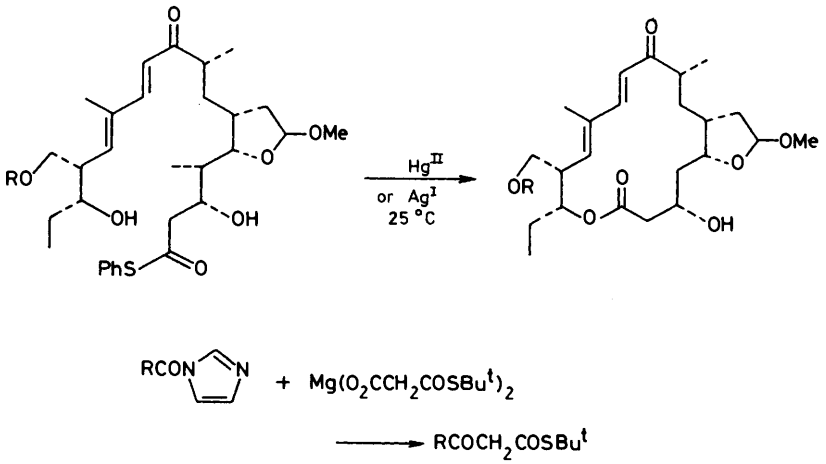
aldehyde or ketone might be a reasonable mechanism for the reactions catalysed by malate and citrate synthases. Such a sequence would involve the formation of lactones, as was first suggested in 1943 by Martius.[17] Although the stereochemistry of the reactions can be accounted for in this way, the lactone intermediates must undergo hydrolysis to obtain the product and this reaction would require catalysis by the enzyme. Thus the synthases would have to exhibit condensing and hydrolysing activity; catalysis of more than one reaction by an enzyme is not

[16] K. T. Douglas, M. Alberz, G. R. Rullo, and N. F. Yaggi, *J. Chem. Soc., Perkin Trans. 2*, 1982, 1675, 1681.

[17] C. Martius, *Hoppe Seyler's Z. Physiol. Chem.*, 1943, **279**, 96.

unusual. The ketene mechanism has also been considered in the context of fatty acid biosynthesis.[18]

The facility with which thiol esters undergo condensation reactions has not passed unnoticed by synthetic chemists. For reactions in which conventional acylations are inefficient, such as the synthesis of macrolides, it has been found that thiol esters provide an efficacious solution[19–24] (Scheme 4). Reactions have been



Scheme 4

successful both with simple alkyl compounds and with highly functionalized molecules such as precursors to antibiotic macrolides. Also, close analogues of the chain-extending unit of fatty acid biosynthesis have been used to construct functionalized carbon chains. Several further synthetic transformations modelled upon other enzyme-catalysed reactions will be described below.

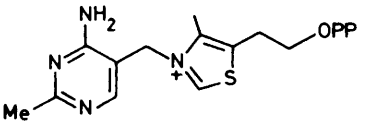
B. Thiamine Pyrophosphate.—In all of the reactions involving thiol esters, the carbonyl group acted as an electrophile; it was therefore easy to construct carbon skeletons with carbonyl groups β to each other. Such reactivity cannot, however, be used to prepare α-dicarbonyl systems. The first chemical reaction to achieve this was the benzoin reaction in which cyanide acts as a catalyst. The condensation is effective because the cyanohydrin can form a carbanion stabilized by the cyano-group and this anion then adds to a second molecule of the aldehyde.

[18] L. Jaenicke and F. Lynen in 'The Enzymes', ed. P. D. Boyer, H. Lardy, and K. Myrback, 2nd edn., Academic Press, New York, 1960, vol. 3, p. 62.
[19] G. E. Wilson, jun. and A. Hess, *J. Org. Chem.*, 1980, **45**, 2766.
[20] S. Masamune, S. Kamata, and W. Schilling, *J. Am. Chem. Soc.*, 1975, **97**, 3515.
[21] S. Masamune, Y. Hayase, W. Schilling, W. K. Chan, and G. S. Bates, *J. Am. Chem. Soc.*, 1977, **99**, 6756.
[22] E. J. Corey and D. J. Brunelle, *Tetrahedron Lett.*, 1976, 3409.
[23] H. Gerlach and A. Thalman, *Helv. Chim. Acta*, 1974, **57**, 2661.
[24] S. Masamune, *Aldrichimica Acta*, 1978, **11**, 23.

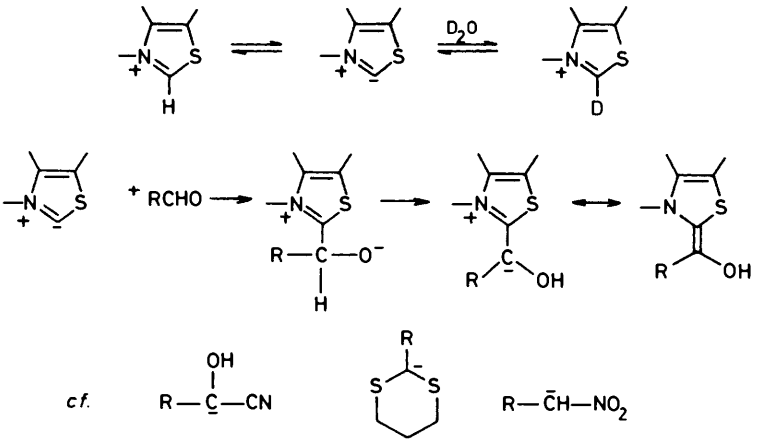
$$PhCHO + CN^- \rightleftharpoons PhCH(OH)CN \rightleftharpoons PhC^-(OH)CN$$

$$PhC^-(OH)CN + PhCHO \rightarrow PhCH(OH)COPh + CN^-$$

It was many years before this catalytic device was developed into a synthetic strategy known as 'umpolung',[25,26] but meanwhile, a good understanding of the related biochemical system had been stimulated by Breslow's experiments on thiamine pyrophosphate.[27] Thiamine pyrophosphate is an *N*-alkylthiazolium salt and Breslow's key observation was that the C-2 proton underwent unusually rapid exchange for deuterium in D_2O solution (Scheme 5). This observation was



Thiamine pyrophosphate TPP

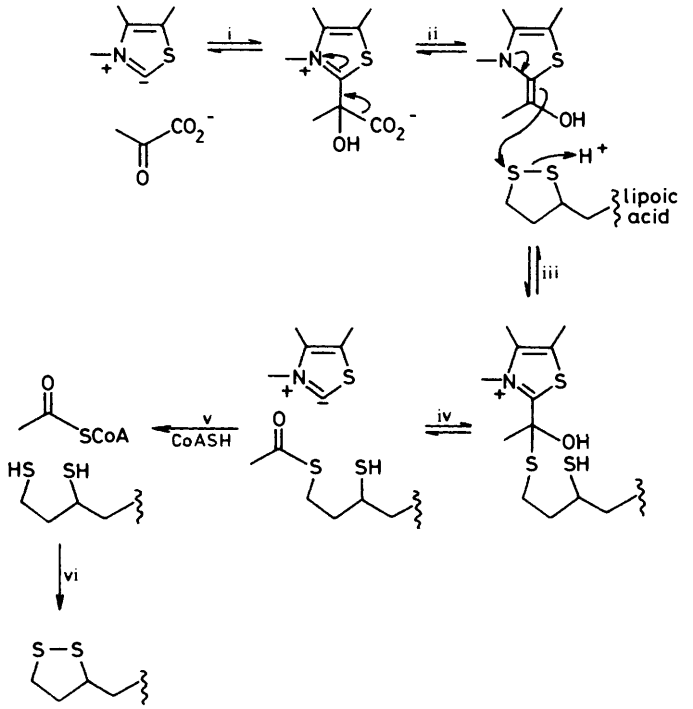


Scheme 5

interpreted as being consistent with the formation of a carbanion at C-2, an ylide in fact, since the molecule also has a positively charged nitrogen atom. Formation of carbanions α to heteroatoms in heterocyclic rings is a normal occurrence but *N*-alkylthiazolium salts are particularly reactive due both to the positive charge and to the sulphur atom. In this way, a nucleophilic intermediate that can add to carbonyl groups can be generated. The parallel between the reactivity of such adducts and synthetic acyl anion equivalents can then be readily seen. In all cases, the carbonyl derivative bears an acidic proton that can yield a stabilized carbanion

[25] Ref. 4, p. 98.
[26] D. Seebach, *Angew. Chem., Int. Edn. Engl.*, 1979, **18**, 239.
[27] R. Breslow, *J. Am. Chem. Soc.*, 1958, **80**, 3719.

(Scheme 5). In bacteria, such intermediates are formed in the synthesis of acetoin, analogous to the benzoin condensation, but a more significant contribution of thiamine pyrophosphate is in the pyruvate dehydrogenase-catalysed conversion of pyruvate into acetyl-CoA, the link between glycolysis and the citric acid cycle. The nature of this enzyme differs widely between organisms but the initial reaction of TPP with pyruvate and its subsequent decarboxylation is common to all of these enzymes (Scheme 6). The success of TPP in this reaction can be readily recognized



Reagents: (i)—(iv), pyruvate decarboxylase; (v), dihydrolipoate transacetylase; (vi), dihydrolipoate dehydrogenase (requires NAD^+ + FAD)

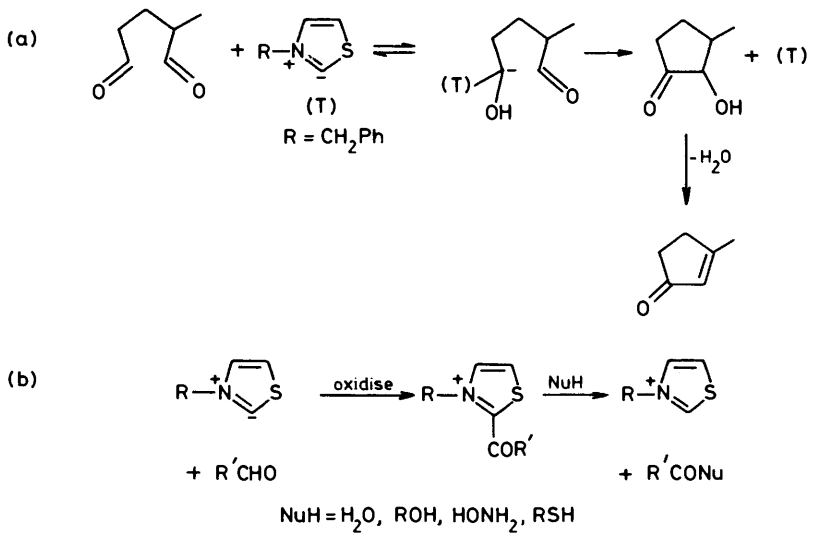
Scheme 6

in its ability to accept electrons from the C—C bond cleaved in decarboxylation. This affords an acetyl anion equivalent, which in the enzyme-catalysed reaction acylates lipoic acid before being transferred to CoA.

Two types of thiazolium derivatives have been exploited in synthesis.[28] Firstly, the direct analogue of the enzymic intermediate has been used in condensation reactions (Scheme 7)[29] and secondly, the oxidation product of this intermediate,

[28] S. Shinkai in 'Enzyme Chemistry, Impact and Applications', ed. C. J. Suckling, Chapman and Hall, London, 1983, chapter 3.

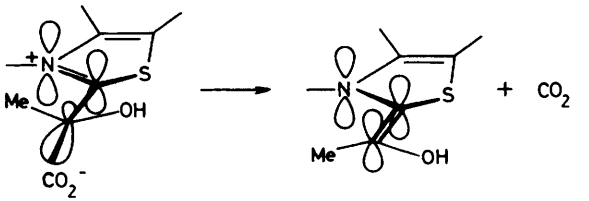
[29] R. C. Cookson and R. M. Lane, *J. Chem. Soc., Chem. Commun.*, 1976, 804.



Scheme 7

a 2-acylthiazolium salt, has been shown to be capable of providing a means for the transformation of an aldehyde into a variety of carboxylic acid derivatives including esters, hydroxamic acids, and thiol esters (Scheme 7).[30,31]

Although TPP effectively stabilizes a carbanion, there is still work for the enzyme to do to catalyse anion formation. The importance of stereoelectronic effects in controlling the courses of non-enzymic reactions is well established[32] and it has been realized that the same factors are important in enzyme-catalysed reactions. One of the first cases to be described in detail concerned PLP, which will be discussed briefly in a moment, but the same arguments apply to TPP.[1] The essence of stereo-electronic control in these cases is that bond breaking to form a stabilized carbanion will be favoured if the bond to be broken lies in the same plane as the *p*-orbitals of the conjugated ring (Scheme 8). The conformation shown is

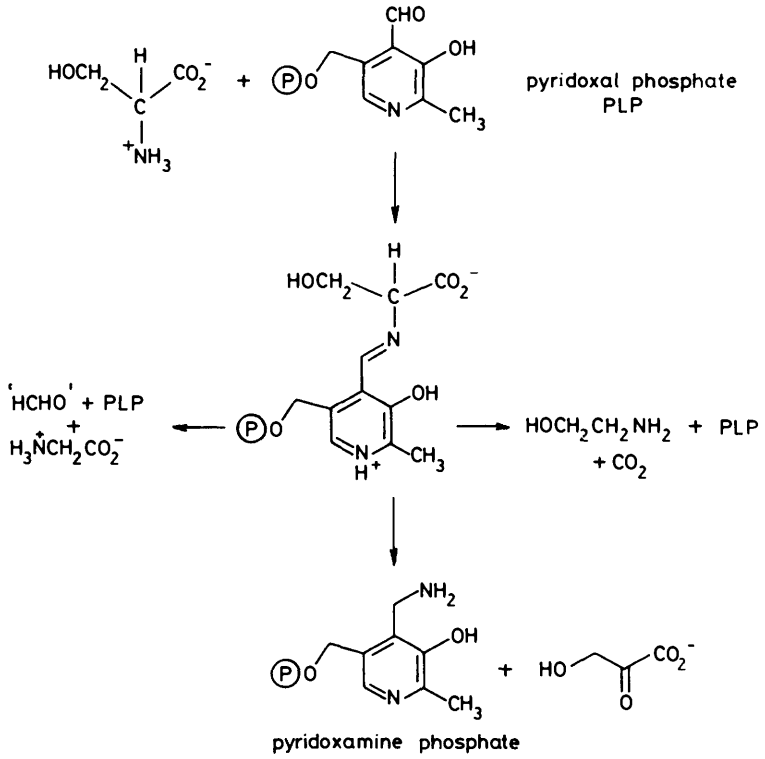


Scheme 8

[30] F. G. White and L. L. Ingraham, *J. Am. Chem. Soc.*, 1962, **84**, 3109.
[31] H. Inoue and K. Higashiwa, *J. Chem. Soc., Chem. Commun.*, 1980, 549.
[32] R. W. Alder, R. Baker, and J. M. Brown, 'Mechanism in Organic Chemistry', Wiley Interscience, New York, 1975, p. 21.

optimal for decarboxylation and the enzyme achieves this arrangement by binding the reactants with a suitably disposed anionic side-chain of an amino-acid at the active site.

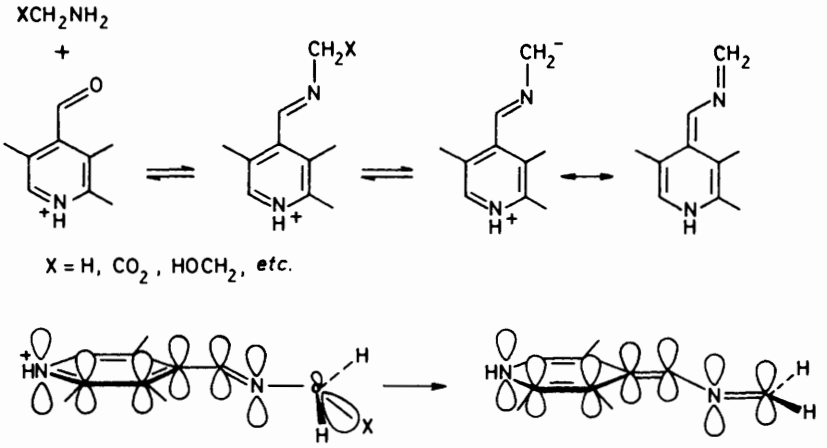
C. Pyridoxal Phosphate.—PLP is involved in a much wider range of reactions than TPP, three of which are illustrated for the amino-acid serine[33] (Scheme 9). In these



Scheme 9

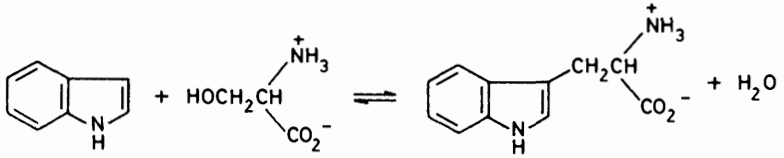
reactions, the carbanion is formed by deprotonation of the first intermediate, an imine (Scheme 10). The enzymes can take advantage not only of stereo-electronic effects to catalyse carbanion formation but can also use them to control the reaction course. As with TPP, the heterocyclic ring stabilizes the carbanion, in this case by delocalization on to the positively charged nitrogen atom. Amino-acids are typical substrates for pyridoxal-dependent enzymes; in some cases, such as serine (Scheme 9), there are three possible bonds that can be broken to give the carbon. Dunathan[34] was the first to point out that which bond is broken can be controlled

[33] Ref. 7, p. 419; ref. 8, p. 82.
[34] H. C. Dunathan, L. Davis, P. G. Kury, and M. Kaplan, *Biochemistry*, 1968, 7, 4532.



Scheme 10

by the conformation of the adduct at the enzyme's active site; the stereo-electronic requirement is the same as with TPP (Scheme 10). Another feature of this group of enzymes that attracted Dunathan's attention was the very close similarity between enzymes with quite different metabolic functions. He envisaged that all PLP-dependent enzymes have evolved from a common progenitor, through slight changes in binding sites, whilst maintaining essentially the same catalytic mechanism. This widely held concept is most attractive chemically because it suggests that PLP-dependent enzymes might be prepared to accept non-natural substrates and be applied to synthesis. One early example of this was the preparation of chiral isotopically labelled glycine using aspartate–pyruvate ammonia transferase:[35] the *pro-R* hydrogen readily undergoes exchange for a heavier isotope in the appropriate medium. Such applications are useful for the synthesis of small quantities of research chemicals, but in Japan, a PLP-dependent enzyme has found use in the industrial synthesis of L-tryptophan (Scheme 11); the

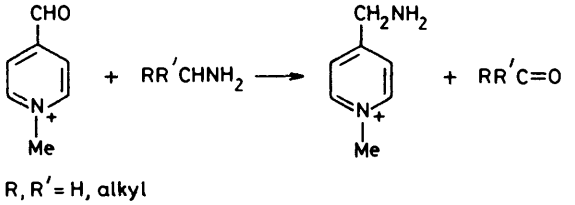


Scheme 11

enzyme was used in immobilized form.[36] A third aspect of PLP chemistry, with respect to synthesis, is the mimicking of the biological process as was discussed for

[35] P. Besmer, Dissertation No. 4435, E.T.H. Zurich, 1970.
[36] S. Fukai, S. Ikeda, M. Fujimura, H. Yamada, and M. Kumagai, *Eur. J. Biochem.*, 1975, **51**, 155.

TPP. Rapoport[37] has stripped PLP of those features necessary for binding to the enzymes (the hydroxymethyl phosphate) and has concentrated the reaction into 4-formyl-*N*-methylpyridinium salts (Scheme 12) thus emphasizing the carbanion



Scheme 12

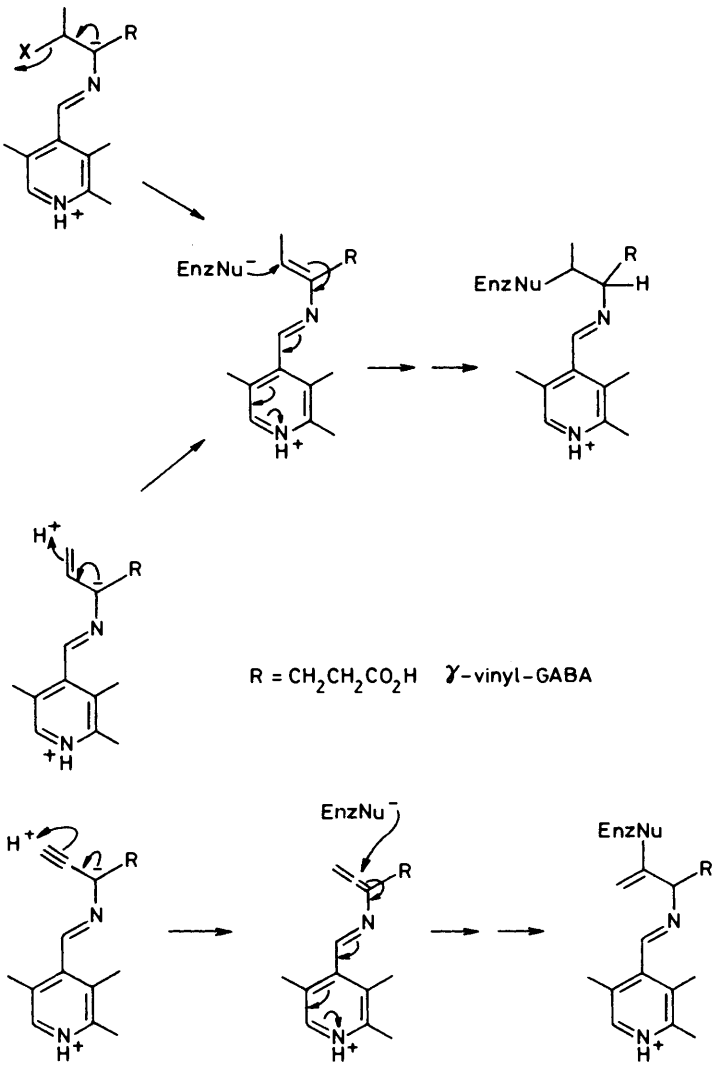
stabilizing features. With this compound, it was possible to promote transaminations of amines to aldehydes and ketones in good yield, a useful reaction because many alternative oxidations of amines to carbonyl compounds employ less selective reagents.

PLP-dependent enzymes also feature in medicinal chemistry. Since they catalyse a great many important metabolic reactions, these enzymes become prime targets for inhibition by drugs in the treatment of pathological conditions. In particular, the concept of mechanism-based, so-called 'suicide' inhibitors, has been extensively investigated.[38] A good understanding of the mechanism of action of an enzyme is an excellent start for the design of an inhibitor and in the case of the PLP enzymes, the ability of the coenzyme to stabilize a carbanion was the focus for attention. It was realised that it might be possible to divert the normal course of reprotonation of the stabilized carbanion through the loss of a good leaving group or through protonation at a more remotely conjugated site (Scheme 13). The products from such reactions are respectively α,β-unsaturated carbonyl equivalents, to which Michael addition may occur, or allenes, which will readily undergo acid-catalysed nucleophilic addition. If the addend is a nucleophilic group from the enzyme's active site, PLP becomes covalently bound to the enzyme which is thereby inhibited. Of the large number of inhibitors of this type, perhaps the most notable is γ-vinyl-GABA, studied by the Merrell group.[39] This compound is an effective and selective inhibitor of GABA-transaminase, the enzyme that initiates the catabolism of the inhibitory neurotransmitter, GABA. Inhibition *in vivo* causes brain GABA levels to be elevated and the compound is undergoing clinical trials as a potential treatment for epilepsy.

3 Radicals and Carbenes

When thinking about organic reaction mechanisms, it is easiest to consider extremes, a fact that has led to much protracted controversy. Thus the advocate of polar mechanisms can easily pay insufficient attention to the possibility that a

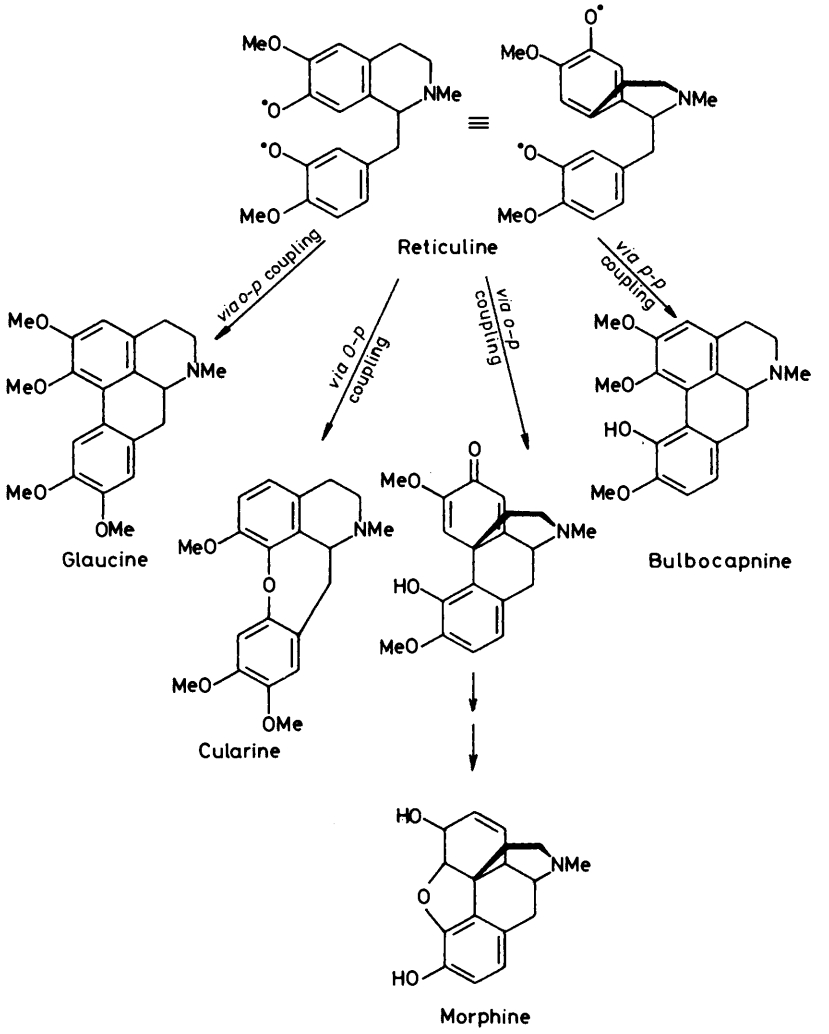
[37] T. F. Buckley and H. Rapoport, *J. Am. Chem. Soc.*, 1982, **104**, 4446.
[38] C. Walsh, *Tetrahedron*, 1982, **38**, 871.
[39] B. Lippert, B. Metcalfe, M. Jung, and P. Casara, *Eur. J. Biochem.*, 1977, **74**, 441.



Scheme 13

radical mechanism might be operating and, of course, *vice versa*. Although polar mechanisms are most frequently discussed in the chemistry of enzymes, a remarkable wealth of radical chemistry exists principally through the involvement of transition metal complexes and through the radical character of the ultimate oxidant, oxygen. The fields in which radical intermediates are important extend well beyond the organic chemist's first preoccupation with such species in enzyme-

catalysed reactions, namely in oxidative coupling reactions in biosynthesis.[40] The oxidation of a phenol to a phenoxy radical, in which the unpaired electron density is delocalized on oxygen and the *ortho* and *para* carbon atoms, allowed the interpretation of a very large number of biosynthetic processes leading to alkaloids and phenolic fungal metabolites (Scheme 14). Many of these reactions can be



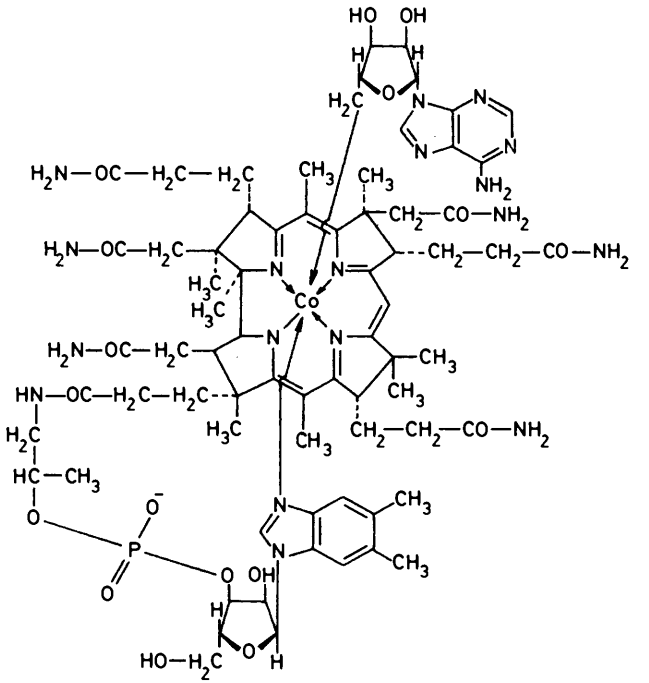
Scheme 14

[40] R. B. Herbert in 'Comprehensive Organic Chemistry', vol. 5, ed. E. Haslam, Pergamon, Oxford, 1979, p. 1068.

reproduced synthetically using transition-metal ion oxidants.[41-43] Interest in radical chemistry in enzyme-catalysed reactions then turned to a remarkable series of rearrangement reactions catalysed by coenzyme B_{12}. More recently, the question of the involvement of radical intermediates in flavin- and nicotinamide coenzyme-mediated reactions has been under active research. Some of the flavin-dependent enzymes are hydroxylation catalysts and parallel research into the chemistry of hydroxylating metalloenzymes, especially cytochrome P-450; additionally, the possibility of carbene intermediates in the latter's reactions has been suggested. A discussion of some aspects of the chemistry of each of these systems follows. However, none of these reactions is more important than the reduction of oxygen at the termini of electron transport chains that complete the energy producing process known as oxidative phosphorylation.[44]

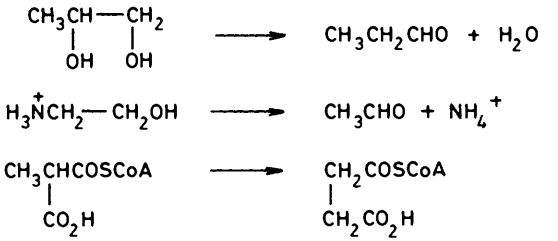
A. Coenzyme B_{12}.—Vitamin B_{12}, or cyanocobalamin, is converted *in vivo* into the enzymically active coenzyme, adenosyl cobalamin that is responsible, amongst other things, for promoting a number of important double 1,2-rearrangements (Scheme 15). Much evidence from both enzymic and model experiments is consistent with the involvement of radical intermediates in B_{12}-mediated reactions[45] but, as is frequently the case with any mechanistic study, direct evidence has been very difficult to obtain. It has, however, been well established that coenzyme B_{12} and related model compounds can exist in three oxidation states. These can be understood as arising formally from heterolytic cleavage of the Co—C bond to give cobalt(I) and an organic cation, heterolytic cleavage to cobalt(II) and a radical or the second possible heterolytic cleavage giving cobalt(III) and a carbanion. Each can be brought about under appropriate conditions but the photolability of the Co—C bond to give cobalt(II) and a radical is a particularly facile reaction. One line of evidence that has led to the belief that radical intermediates are likely in the enzyme-catalysed reactions is the study of the stereochemical course of the rearrangement reactions using isotopic labels. The reaction catalysed by ethanolamine ammonia lyase is an interesting case in which racemization of the substrate takes place; an unusual event in an enzyme whose metabolic function is not racemization[46,47] (Scheme 16). The results were consistent with a mechanism in which homolysis of the Co—C bond leads to an adenosyl radical, which abstracts a hydrogen atom from the substrate to initiate rearrangement. The reaction is terminated by the reverse abstraction of a hydrogen atom from the adenosyl methyl group by the substrate but evidently, before this happens, there is sufficient time for the substrate to racemize by rotation. There has been much effort to understand the detailed nature of the rearrangement step and

[41] D. H. R. Barton and G. W. Kirby, *J. Chem. Soc.*, 1962, 806.
[42] M. A. Schwartz and R. A. Holton, *J. Am. Chem. Soc.*, 1970, **92**, 1090.
[43] M. A. Schwartz and I. S. Mami, *J. Am. Chem. Soc.*, 1975, **97**, 1239.
[44] B. G. Malmstrom, *Ann. Rev. Biochem.*, 1982, **51**, 21.
[45] B. T. Golding in Comprehensive Organic Chemistry', vol. 5, ed. E. Haslam, Pergamon, Oxford, 1979, 549.
[46] J. Retey, C. J. Suckling, D. Arigoni, and B. M. Babior, *J. Biol. Chem.*, 1974, **249**, 6359.
[47] D. Gani, O. C. Wallis, and D. W. Young, *Eur. J. Biochem.*, in press.



Coenzyme B_{12}

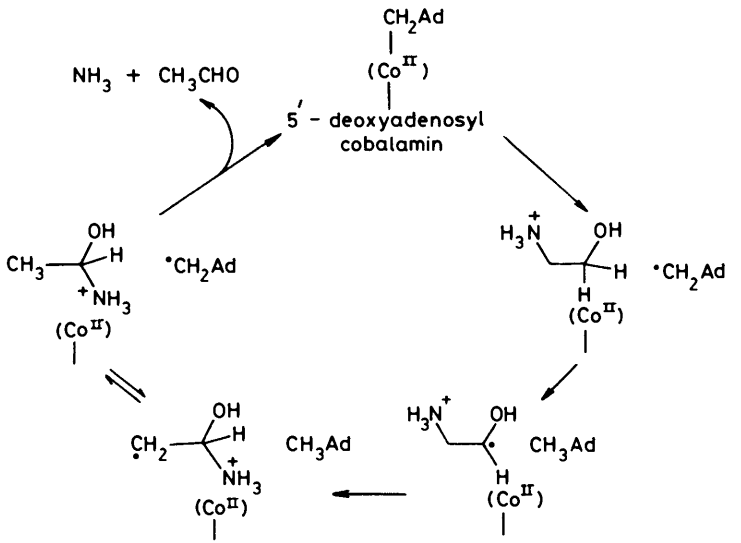
Some rearrangements catalysed by coenzyme B_{12}-dependent enzymes



Scheme 15

cobalt–π-complexes have been popular intermediates. More subtle suggestions involving electrocyclic reactions of the coenzyme ring have also been made.[48] Whilst not attracting great support, the proposition of this alternative makes it clear that a satisfactory understanding of these reactions has not yet been reached.

[48] E. J. Corey, N. J. Cooper, and M. L. H. Green, *Proc. Nat. Acad. Sci. USA.*, 1977, **74**, 811.

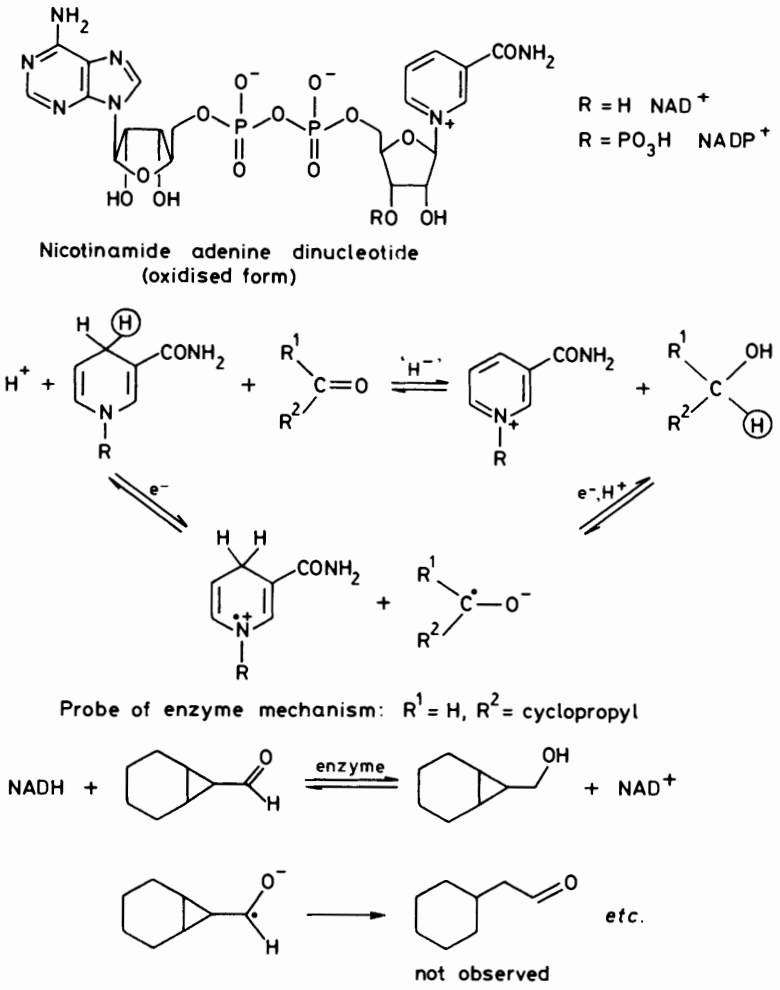


Scheme 16

B. Reactions Mediated by Flavin and Nicotinamide Coenzymes.—The heterocyclic coenzymes containing nicotinamide or flavin systems are the mainstays of biological oxidation reactions at the level of organic substrates. Consequently there has been a great deal of interest in the mechanisms of action of these compounds in enzyme catalysed reactions. Additionally, chemists have been intrigued by the possibility of mimicking the biological reactivity with model systems.[28] Although this pursuit has led to much interesting chemistry, it has also fermented controversy concerning the mechanisms of action of the coenzymes at the enzymes' active site. The centre of the discussion has concerned the possible intermediacy of radicals and a few words on the current position for nicotinamide and flavin coenzymes follows.

In the case of nicotinamide coenzymes, two of the chief candidate mechanisms are summarized in Scheme 17. There have been several reliable reports of one-electron transfer reactions from reduced nicotinamide coenzymes (NADH) analogues to sufficiently strong oxidants such as ferricyanide but the situation has been confused by a large number of studies of reactions with but weakly oxidizing substrates that have been interpreted as indicating radical intermediates.[49] Very few experiments, however, have addressed themselves to the more difficult task of probing the enzyme's mechanism itself. The best way to tackle this problem was to use a molecule that would report the intermediacy of radical intermediate and

[49] For an extensive list of references and critical discussion see M. F. Powell and T. C. Bruice, *J. Am. Chem. Soc.*, 1983, **105**, 1014.



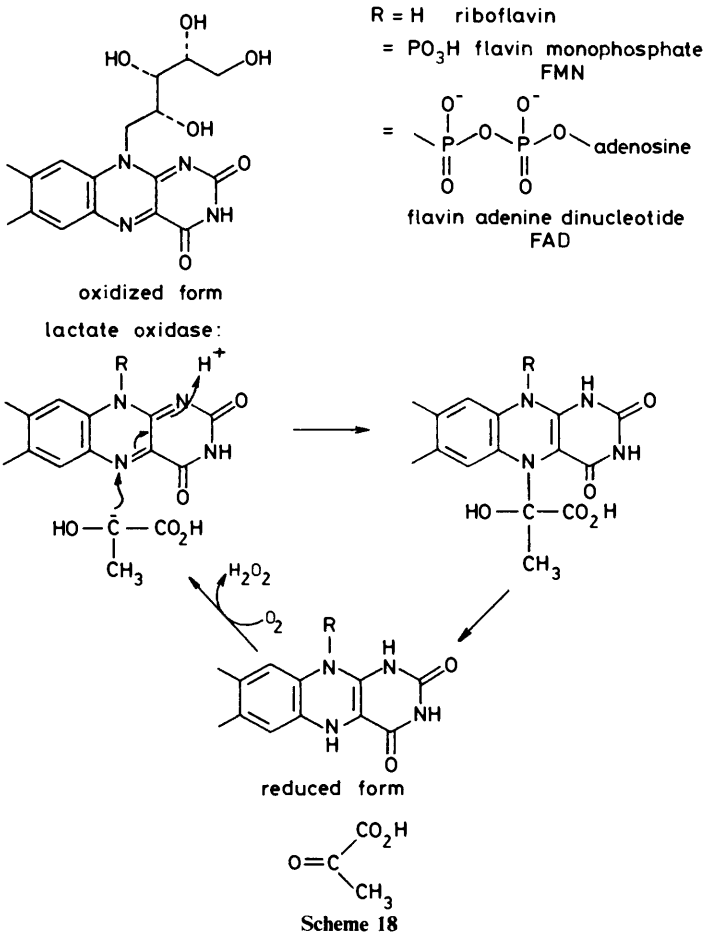
Scheme 17

this has been done for alcohol dehydrogenase[50] and for lactate dehydrogenase.[51] The principle of the probe is that if a radical forms, the cyclopropane ring will undergo ring opening which can be detected by isolating the products of the reaction. In neither case was any ring opening found, and related model reactions showed the same behaviour.[51,52] The current concensus is that nicotinamide-

[50] I. MacInnes, D. C. Nonhebel, S. T. Orszulik, and C. J. Suckling, *J. Chem. Soc., Chem. Commun.*, 1982, 121.
[51] D. C. Nonhebel, S. T. Orszulik, and C. J. Suckling, *J. Chem. Soc., Chem. Commun.*, 1982, 1146.
[52] J. C. T. van Niel and U. K. Pandit, *J. Chem. Soc., Chem. Commun.*, 1983, 149.

mediated oxidations and reductions take place through hydride transfer mechanisms.

With flavins, the situation is more complex because these molecules are very effective one-electron oxidants and reductants as well as undergoing hydride-like two-electron reactions. As Walsh has aptly put it, flavin coenzymes are at the crossroads of biological redox chemistry.[53] One further major difference between flavins and nicotinamide coenzymes is that the reduced form of the former can be oxidized directly by oxygen whereas the latter cannot. In fact, many flavin-mediated reactions involve oxygen as a co-substrate. In some cases such as amino-acid oxidases and hydroxy-acid oxidases, there is good evidence for the formation of carbanions at the substrates, although the nature of the oxidation step is still uncertain (Scheme 18). For substrates lacking the anion-stabilizing

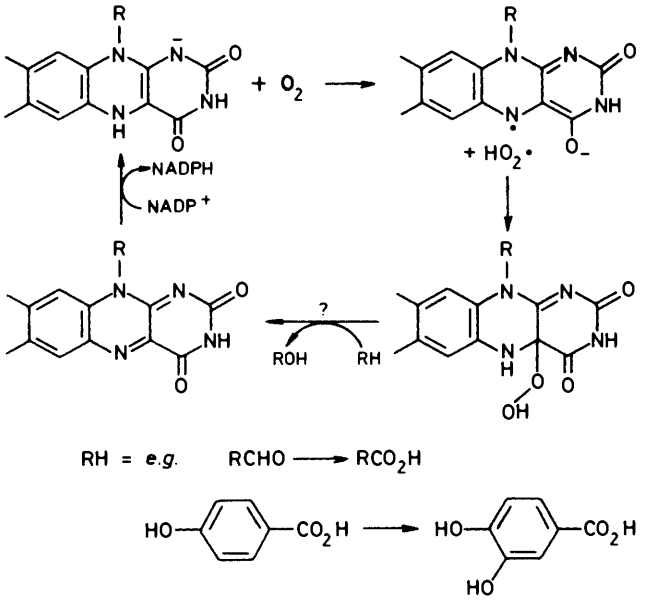


Scheme 18

[53] C. Walsh, *Acc. Chem. Res.*, 1980, **13**, 148.

carbonyl group such as amines in amine oxidases, radical mechanisms may be involved. Indeed Bruice has obtained evidence from model studies that the oxidation of carbanions by flavins takes place through one-electron steps,[54,55]

An alternative reaction of flavins with oxygen leads to incorporation of one atom of the dioxygen molecule into the substrate. Oxygen activation is thought to proceed through electron transfer from the reduced flavin to oxygen followed by combination of the radical pair. This leads to a 4*a*-hydroperoxyflavin which is the prime candidate for the biological hydroxylating agent (Scheme 19).[53] Bruice has

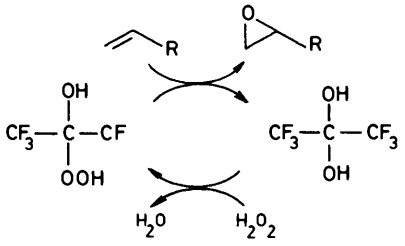


Scheme 19

shown that the properties of this molecule belong to the same general series as alkyl hydroperoxides and peracids;[56] the mechanism by which subsequent hydroxylations take place, however, are not yet established. Arguments have been presented for direct oxygen transfer,[56,57] for radical recombinations,[54] and for *N*-5-oxides.[58] In such discussions, it is important never to forget the nature of the substrate and it is highly probable that the mechanism of hydroxylation of aromatic compounds may be quite different from the oxidations of aldehydes and ketones to carboxylic acid derivatives.[59] Nevertheless, the activity in the chemistry

[54] M. Novak and T. C. Bruice, *J. Am. Chem. Soc.*, 1977, **99**, 8079.
[55] T. C. Bruice, *Acc. Chem. Res.*, 1980, **13**, 246.
[56] T. C. Bruice, *J. Chem. Soc., Chem. Commun.*, 1983, 14.
[57] B. Entsch, D. Ballou, and V. Massey, *J. Biol. Chem.*, 1976, **251**, 2550.
[58] J. W. Frost and W. H. Rastetter, *J. Am. Chem. Soc.*, 1981, **103**, 5242.
[59] C. Walsh, 'Enzymatic Reaction Mechanisms', Freeman, San Francisco, 1979, chapters 11 and 12.

of flavins has led to the development of a useful mild epoxidizing agent that has some similarities with the reactivity of flavins. As Bruice has pointed out, the oxidizing ability of flavin 4*a*-hydroperoxides can be ascribed chiefly to the low pK_a of the hydroperoxide (9.1—9.5). A similar situation exists in the hydroperoxyhydrate of hexafluoroacetone[60,61] and this property has been harnessed for an effective synthetic reagent (Scheme 20).



CH_2ClCH_2Cl reflux in presence of Na_2HPO_4 anhydrous

Scheme 20

C. Reactions Mediated by Cytochromes P-450.—Cytochrome P-450 is the name commonly given to a class of haemoproteins that catalyses hydroxylation reactions. A wide variety of organic molecules can act as substrates, some endogenous and some foreign to the organism, including steroids, smaller alicyclic hydrocarbons, benzenoid, and polycyclic hydrocarbons. The enzyme operates by a cyclic mechanism (Figures 2 and 3). Self destruction as a result of generating reactive hydroxylating species is prevented by the requirement that a molecule of substrate binds to the active site before oxygen. The stoicheiometry of the cycle and the shunt by which organic hydroperoxides can short circuit it are well established but the nature of the hydroxylating species is still a topic of active research. As was discussed for the flavin-dependent enzymes, there is a great variety of substrates for cytochromes P-450 and it would not be surprising, therefore, if the mechanisms of action reflected the reactivity of the substrates as well as the catalytic properties of the enzyme. Nevertheless, an essential part of all P-450 species appears to be a thiolate ligand from the protein which co-ordinates to an axial position of the iron opposite the oxygen. It is most probable that this excellent donor ligand has the job of stabilizing an electrophilic hydroxylating agent generated at the iron.

The connection between cytochromes P-450 and radical and carbene intermediates was first discussed by Hamilton[62] who suggested that the potent hydroxylating ability of these enzymes might be due to the generation of a highly electrophilic oxygen species, oxene, which would insert into a carbon–hydrogen

[60] R. P. Heggs and B. Ganem, *J. Am. Chem. Soc.*, 1979, **101**, 2486.
[61] A. J. Bileski, R. P. Heggs, and B. Ganem, *Synthesis*, 1980, 810.
[62] G. A. Hamilton in 'Molecular Mechanisms of Oxygen Activation', ed. O. Hayaishi, Academic Press, New York, 1974, p. 405.

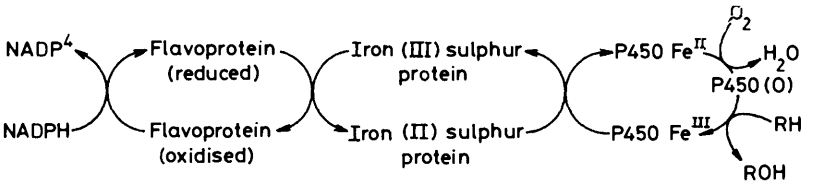


Figure 2 *The cytochrome* P-450 *system of adrenal steroidogenic tissue*

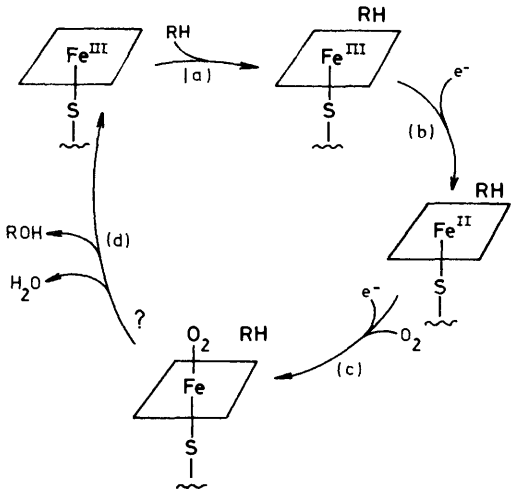
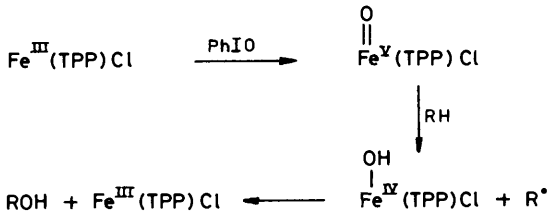


Figure 3 *Proposed mechanism for cytochrome* P-450. (a) RH *binds to low-spin* Fe^{III} P-450, *converting it into a high-spin complex.* (b) *One electron enters from the* P-450 *reductase, giving an* Fe^{II} *state.* (c) *Oxygen binds and a second electron enters, forming the formal* $Fe^{II}—O_2^-$ *complex.* (d) *The active form of oxygen is generated, hydroxylation takes place and the products,* ROH *and water, diffuse off the complex, leaving it in its initial state*

bond in an analogous manner to singlet carbenes.[63] This suggestion, although attractive, has not received much experimental support and the balance of evidence now seems to favour radical pathways, especially for aliphatic hydroxylation. The first results pointing strongly in this direction were from model studies. Following earlier work showing that metalloporphyrins are potent autoxidation initiators, Groves discovered that tetraphenylporphyrin–metal complexes together with iodosobenzene as a source of oxygen have a lot in common with the enzymes

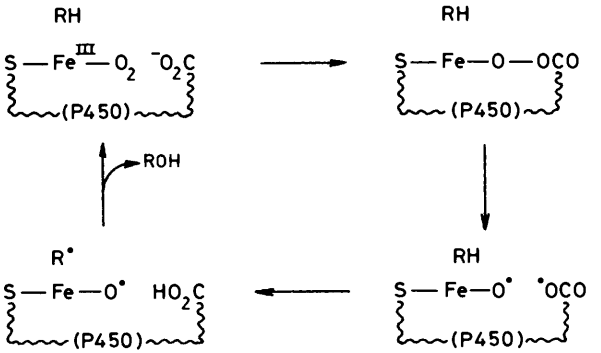
[63] W. Kirmse, 'Carbene Chemistry', 2nd edn., Academic Press, New York, 1971.

themselves.[64–67] He has been able to establish that the complex formed after oxygen is transferred to the iron from iodosobenzene is able to abstract hydrogen from a substrate yielding a radical which then recombines with the hydroxy-group co-ordinated to iron as if on a rebound (Scheme 21). The characteristics of the



Scheme 21

reactions of this system with such substrates as alkenes leading to allylic rearrangements and cyclopropanes leading to ring opening are also consistent with the intermediacy of radicals. A related mechanism may operate with the enzymes themselves and the observation of a substantial kinetic isotope effect in the oxidation of norbornane is consistent with this view.[68,69] However, there have been strong suggestions that in the reaction with oxygen itself, a complexed acyl hydroperoxide is an intermediate (Scheme 22). Homolytic cleavage of this complex

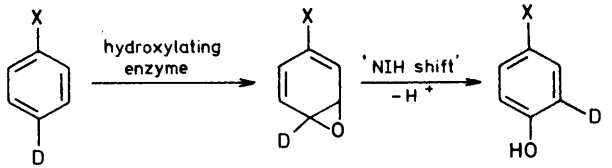


Scheme 22

[64] J. T. Groves in 'Metal Ion Activation of Dioxygen', ed. T. G. Spiro, Wiley, New York, 1980, p. 125.
[65] J. T. Groves, T. E. Nemo, and R. S. Myers, *J. Am. Chem. Soc.*, 1979, **101**, 1032.
[66] J. T. Groves, W. J. Kruger, jun., and R. C. Haushalter, *J. Am. Chem. Soc.*, 1980, **102**, 6375.
[67] J. T. Groves, R. C. Haushalter, M. Nakamura, T. E. Nemo, and B. J. Evans, *J. Am. Chem. Soc.*, 1981, **103**, 2884.
[68] J. T. Groves, G. A. McClusky, R. E. White, and M. J. Coon, *Biochem. Biophys. Res. Commun.*, 1978, **81**, 154.
[69] M. J. Coon and R. E. White in 'Metal Ion Activation of Dioxygen', ed. T. G. Spiro, Wiley, New York, 1980, p. 73.

leads to two radicals at the active site, one of which abstracts hydrogen leaving the complexed oxygen able to bond to the substrate radical produced.[70–72] This pattern of reactivity seems to be consistent with the hydroxylation of cumene, camphor, and cyclohexane and also indirectly with the oxidation of cyclopropyl benzene to benzoic acid in which three sequential oxidation steps seem to occur.[73]

Aromatic compounds offer more mechanistic ambiguities than aliphatic substrates as can be seen by the effect of exchanging oxygen for sulphur in the demethylation of arylmethyl ethers.[74,75] It has been found that a large primary hydrogen-isotope effect accompanies the demethylation of a series of substituted anisoles[75,76] and this result has been interpreted to indicate rate-determining hydrogen abstraction from the methyl group. However the analogous series of thioethers showed no such isotope effect. Instead, a correlation between the one-electron oxidation potential of the thioether and the rate of demethylation was observed. In this case, it was argued that the rate-determining step involves electron transfer from substrate to the haem complex. There is also evidence that aromatic hydroxylation occurs through addition to the aromatic ring and not through abstraction or insertion mechanisms (Scheme 23). The well known NIH



Scheme 23

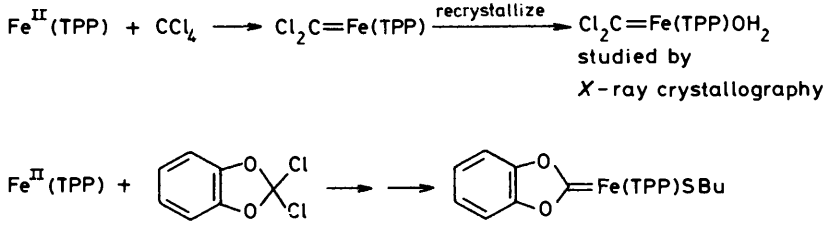
shift has been a major technique for diagnosing addition-rearrangement mechanisms: as indicated in Scheme 23, rearrangement of an intermediate epoxide leads to substantial retention of isotopic label (limited by the kinetic isotope effect). This behaviour is characteristic of cytochromes P-450 and of a few model systems in which a strong oxidant is present.[77] The metabolism of warfarin *in vivo* in rats is thought to proceed through a mechanism of this type.[78]

One of the consequences of the generation of radical intermediates by cytochromes P-450 at their active sites is that side reactions in which the radicals attack the porphyrin ligand can take place. This phenomenon has been observed

[70] R. E. White, S. G. Sligar, and M. J. Coon in 'Biochemistry, Biophysics and Regulation of Cytochrome P-450', ed. J.-A. Gustaffson, Elsevier North Holland, 1980, p. 307.
[71] R. C. Blake II and M. J. Coon, in ref. 70, p. 315.
[72] S. G. Sligar, M. Besman, M. Gelb, P. Gould, D. Heimbrook, and D. Pearson, in ref. 70, p. 379.
[73] K. E. Suckling, C. G. Smellie, I. E. Ibrahim, D. C. Nonhebel, and C. J. Suckling, *FEBS Lett.*, 1982, **145**, 179.
[74] Y. Watanabe, T. Iyanagi, and S. Oae, *Tetrahedron Lett.*, 1982, **23**, 533.
[75] Y. Watanabe, S. Oae, and T. Iyanagi, *Bull. Chem. Soc. Jpn.*, 1982, **55**, 188.
[76] J. R. Lindsay Smith, R. E. Piggott, and P. R. Sleath, *J. Chem. Soc., Chem. Commun.*, 1982, 55.
[77] L. Castle and J. R. Lindsay Smith, *J. Chem. Soc., Chem. Commun.*, 1978, 704.
[78] E. D. Bush and W. F. Trager, *Biochem. Biophys. Res. Commun.*, 1982, **104**, 626.

with a variety of substrates including substituted cyclopropylmethylamines.[79,80] A further ramification of this reaction concerns the oxidation of halogen-containing anaesthetics and related compounds in the liver, a major location of cytochrome P-450.[81] The generation of trichloromethyl radical by microsomes containing cytochrome P-450 and in rat liver has been demonstrated[82] but such results by no means prove a relationship between the formation of radicals and liver damage. Nevertheless, there seems little doubt that the adventitious generation of radicals *in vivo* is a major cause of tissue damage and the relationship between lipid peroxidation and ageing has received considerable attention.[81]

Although the reactions of cytochrome P-450 discussed above have been interpreted chiefly in terms of radical mechanisms and not oxenoid intermediates, there is conclusive evidence for the formation of carbene complexes related to cytochrome P-450 from *X*-ray analysis (Scheme 24).[83] This phenomenon led



Scheme 24

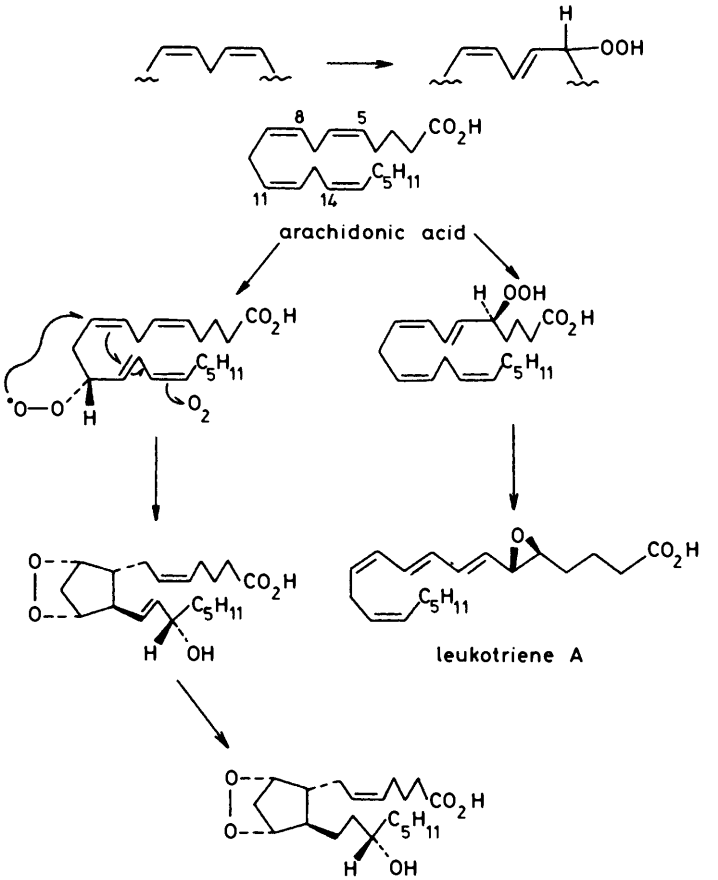
Mansuy and Ullrich to investigate the cause of the synergism of 1,3-benzodioxoles with insecticides.[84] They found that a dichlorobenzodioxole reacts with tetraphenylporhinatoiron(II) (Scheme 24) and, on addition of butyl thiolate, a spectrum resembling that of the enzyme treated with the synergist resulted. This reaction causes inhibition of the enzyme. Since hydroxylation by cytochrome P-450 is a major detoxification route for foreign compounds in many organisms, the synergism of the benzodioxoles could well be due to the blocking of the insects' detoxification pathways.

In view of the extensive hydroxylating ability of cytochromes P-450 and other metal-containing oxygenases, it is not surprising that attempts have been made to mimic their reactivity with simpler chemical models.[85] One goal of research has been to match the catalytic efficiency of the enzymes and this has best been attained

[79] R. P. Hanzlik and R. H. Tullman, *J. Am. Chem. Soc.*, 1982, **104**, 2048.
[80] T. L. Macdonald, K. Zirvi, L. T. Burka, P. Peyman, and F. P. Guengerich, *J. Am. Chem. Soc.*, 1982, **104**, 2050.
[81] 'Free Radicals in Biology', vol. 4, ed. W. A. Pryor, Academic Press, New York, 1980.
[82] P. B. McCoy, T. Noguchi, K.-L. Fong, E. K. Lui, and J. L. Poyer, in 'Free Radicals in Biology', vol. 4, ed. W. A. Pryor, Academic Press, New York, 1980, p. 157.
[83] D. Mansuy, M. Lange, J.-C. Chottard, J. F. Bartoli, B. Chevrier, and R. Weiss, *Angew. Chem., Int. Edn. Engl.*, 1978, **17**, 781.
[84] D. Mansuy, J. P. Battioni, J.-C. Chottard, and V. Ullrich, *J. Am. Chem. Soc.*, 1979, **101**, 3971.
[85] T. Matsuura, *Tetrahedron.*, 1977, **33**, 3971.

using manganese tetraphenylporphyrin complexes as catalysts for hydroxylations[86] and epoxidations.[87,88] Alternatively, emphasis has been placed upon attaining enzyme-like regioselectivity in hydroxylation.[89,90] The achievement of high selectivity with high catalytic efficiency has yet to be reached for the latter.

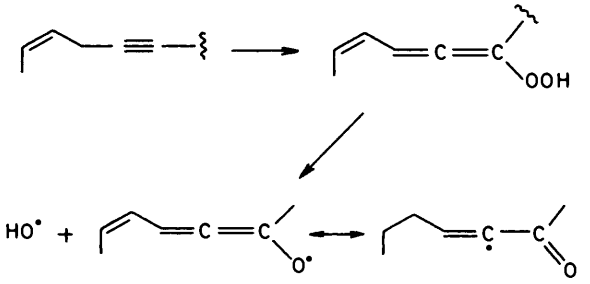
D. Reactions Catalysed by other Haemoproteins.—A second group of haemoproteins in which oxygen is caused to react with organic substrates to generate radicals is concerned in the formation of hydroperoxides. A particularly



Scheme 25

[86] I. Tabushi and N. Koga, *J. Am. Chem. Soc.*, 1979, **101**, 6456.
[87] E. Guilmet and G. Meunier, *Tetrahedron Lett.*, 1980, 4449.
[88] D. Mansuy, M. Fontcave, and J.-F. Bartoli, *J. Chem. Soc., Chem. Commun.*, 1983, 253.
[89] C. J. Suckling, *Ind. Eng. Chem., Prod. Res. Dev.*, 1981, **20**, 434.
[90] C. J. Suckling, *J. Chem. Res.*, 1981, (S) 280, (M) 3279—3291.

important reaction is the conversion of *cis,cis*-1,4-pentadienes, a common feature of polyunsaturated fatty acids, into the *cis,trans* conjugated hydroperoxide (Scheme 25). Such a reaction is characteristic of a radical mechanism and evidence for the intermediacy of radicals *in vivo* has been obtained.[82] The significance of these reactions lies in their participation in the biosynthesis of prostanoids including prostaglandins, prostacyclins, thromboxanes, and leukotrienes (including the so-called slow-reacting substances of anaphylaxis). All of these compounds are derived from oxidation reactions of arachidonic acid (Scheme 25), which contains two pairs of *cis* double bonds. Oxidation at C-5 leads to leukotrienes whereas oxidation at C-11 leads to prostaglandins. Since arachidonate metabolites have a wide range of biochemical actions—including inflammation, the control of blood platelet aggregation, and the control of the reproductive cycle—chemical manipulation of the pathway has become an attractive proposition for medicinal chemists.[91,92] To illustrate an approach to manipulating these biosynthetic pathways, Corey's extensions of his original leukotriene synthesis are relevant.[93,94] The design strategy was to generate specific inhibitors for either prostaglandin or the leukotriene branch by replacing one of the double bonds by an acetylene. In the hydroperoxide-forming reaction, an allylic hydroperoxide would result at the site determined by the enzyme. If this site held an acetylene, an allenic hydroperoxide would form instead, fragmentation of which would lead to reactive radicals that would inhibit the enzyme (Scheme 26). It was



Scheme 26

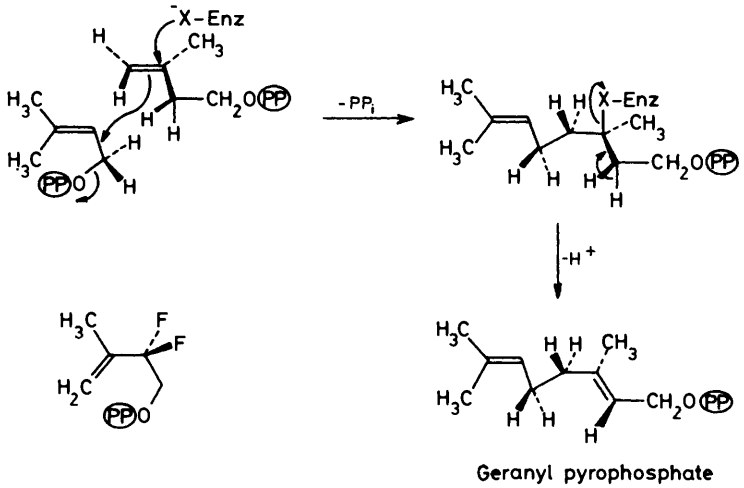
found that both 11,12- and 14,15-dihydroarachidonic acids were potent inhibitors of prostaglandin biosynthesis, whereas the 5,6-isomer inhibited leukotriene biosynthesis selectively, as would be expected from the mechanism.

4 Carbocations

Whereas the scope of organic-radical intermediates in enzyme-catalysed reactions is large, chemistry relating to carbocations chiefly concerns the biosynthesis of

[91] B. Hesp and A. Willard in 'Enzyme Chemistry, Impact and Applications', ed. C. J. Suckling, Chapman and Hall, London, 1984, chapter 5.
[92] J. Ackroyd and F. Scheinemann, *Chem. Soc. Rev.*, 1982, **11**, 321.
[93] E. J. Corey and H. Park, *J. Am. Chem. Soc.*, 1982, **104**, 1750.
[94] E. J. Corey and J. E. Munroe, *J. Am. Chem. Soc.*, 1982, **104**, 1752.

isoprenoid compounds.[95,96] In this field, a number of striking rearrangement processes have been shown to occur with a high degree of stereospecificity and some are shown in Scheme 27. A great deal of elegant chemistry using isotopically



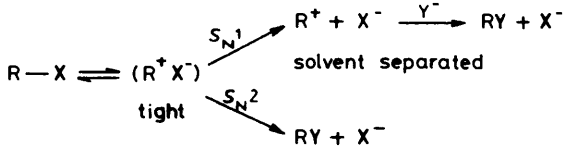
Scheme 27

labelled molecules has established the stereochemical course of such reactions. Since most of the stereospecific reactions then known related to S_N2 and $E2$ reactions, it was natural for chemists to formulate such biosynthetic reactions in similar terms. For the interconversion of isopentenyl and dimethyl allyl pyrophosphates (Scheme 27), this interpretation took the form of an enzyme nucleophile (SMe) that both stabilized an incipient positive charge and then acted as a good leaving group. This, and similar rationalizations, found wide acceptance. No experimental evidence was available to test the hypothesis, however, largely because of difficulties in obtaining sufficiently pure samples of the appropriate enzymes. The first experimental questioning of the nucleophile hypothesis came when Poulter[97,98] showed that the behaviour of fluorinated analogues of isopentenyl pyrophosphate (see Scheme 27) and other isoprenoids was not consistent with the addition–elimination mechanism demanded by the participation of a nucleophilic group on the enzyme's active site. For example, if the analogue shown in Scheme 27 underwent reaction by the hypothesized pathway, loss of a proton to eliminate the enzymic nucleophile would be impossible because of fluorine substitution. Hence the compound should act as an

[95] J. W. Cornforth, *Quart. Rev.*, 1969, 125.
[96] J. R. Hanson in 'Comprehensive Organic Chemistry', vol. 5, ed. E. Haslam, Pergamon, Oxford, 1979, p. 989.
[97] C. D. Poulter, E. A. Marsh, J. C. Argyle, O. J. Muscio, and H. C. Rilling, *J. Am. Chem. Soc.*, 1979, **101**, 6761.
[98] C. D. Poulter and H. C. Rilling, in 'Biosynthesis of Isoprenoid Compounds', vol. 1, ed. J. W. Porter and S. L. Spurgeon, Wiley Interscience, New York, 1981, p. 161.

irreversible inhibitor. Although the fluorinated analogues bind to the enzyme well, no irreversible inhibition was observed. On the basis of this and other evidence, Poulter argues that the addition–elimination mechanism should be abandoned.

Many years earlier, some provocative papers by Sneen[99] advocated the idea that even S_N2 reactions take place not by concerted processes but through tight ion pairs, which kinetically are equivalent to the undissociated substrate (Scheme 28).



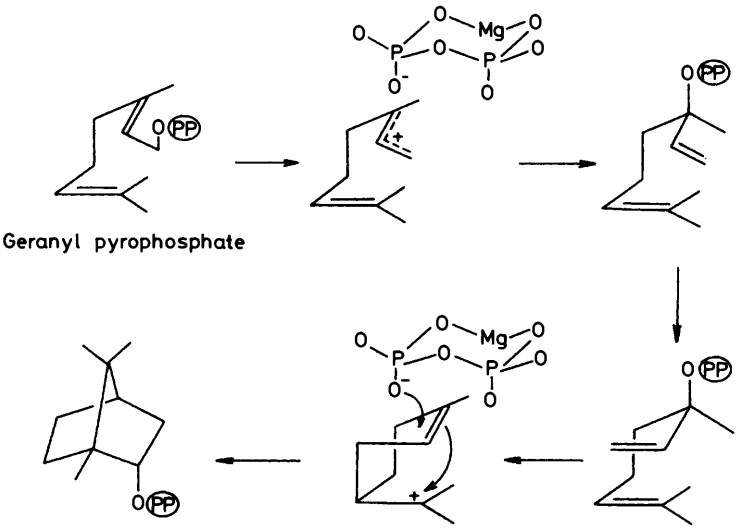
Scheme 28

The extension to the S_N1 mechanism is obvious. One attractive feature of this point of view is that the transition between S_N1 and S_N2 behaviour can be envisaged as reflecting the tightness of the ion pairing, a situation that would be strongly dependent upon the reaction conditions. Indeed with regard to cyclization reactions in terpene biosynthesis, evidence has been presented to suggest that a spectrum of cyclization mechanisms from concerted to ionic through degrees of ion pairing is possible.[100]

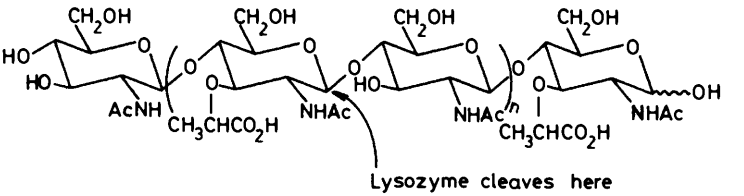
The enzymic counterpart of these experiments has begun to emerge recently, chiefly through the work of Cane.[101,102] With the aid of ^{18}O labels, his group has established that the enzyme-catalysed cyclization of several acyclic isoprenoids occurs without loss of the label (Scheme 29). Indeed in some cases, the labelled oxygen does not even exchange by rotation about the P—O bond. Thus the best current interpretation of these results is that enzyme-catalysed cyclizations take place through a series of tight ion pairs. The phosphate leaving-group remains, presumably co-ordinated to magnesium at the active site, and the rearrangement takes place with the positive charge remaining virtually within bonding distance of the phosphate. This obviates the need for an additional electron-donating group to stabilize the cation in a most economical fashion.

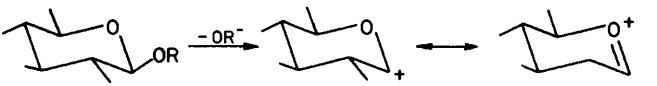
There are, however, cases in which the structure of the enzyme has a specific capability for stabilizing cationic intermediates. The classic example of this phenomenon concerns the enzyme lysozyme which hydrolyses amino-polysaccharides (Scheme 30). Cationic mechanisms for such reactions are favoured by the ability of the adjacent oxygen atom to stabilize the positive charge,[103] but even a delocalized cation must have a counter ion. In lysozyme, *X*-ray crystallography has shown that an aspartate residue is positioned close to the site

[99] R. A. Sneen and J. W. Larsen, *J. Am. Chem. Soc.*, 1969, **91**, 362, 6031.
[100] C. D. Poulter and C. H. R. King, *J. Am. Chem. Soc.*, 1982, **104**, 1420, 1422.
[101] D. E. Cane in 'Enzyme Chemistry, Impact and Applications', ed. C. J. Suckling, Chapman and Hall, London, 1984, chapter 7.
[102] D. E. Cane, A. Saito, R. Croteau, J. Shasko, and M. Felton, *J. Am. Chem. Soc.*, 1982, **104**, 7274.
[103] L. Hough and A. C. Richardson in 'Comprehensive Organic Chemistry', vol. 5, ed. E. Haslam, Pergamon, Oxford, 1979, p. 714.



Scheme 29





Scheme 30

of generation of positive charge in hydrolysis.[104] The electrostatic interaction contributes to catalysis by this enzyme. A quantitative estimate of the catalytic advantage of such a charge neutralization has been obtained by Sinnott[105] who studied the hydrolysis of glycosylpyridinium salts by β-galactosidase. These reactions proceed unambiguously through an S_N1 pathway with no covalent participation by the enzyme. Compared with the non-enzymic reaction, β-galactosidase causes a rate enhancement of 10^4—10^5 due to charge neutralization.

[104] A. Warshel, *Proc. Nat. Acad. Sci. USA.*, 1978, **75**, 7250.
[105] C. C. Jones, M. L. Sinnott, and L. J. Souchard, *J. Chem. Soc., Perkin Trans. 2*, 1977, 1191.

5 Transition-state Stabilization

At the beginning of this article, I reminded readers about the relationships between the stabilities of transition states and reactive intermediates. Since this relationship has proved a most fruitful one in the chemistry of enzymes, especially their inhibitors, some discussion is worthwhile. The first explicit connection between transition-state structure and enzymic catalysis was made by Pauling[106] who extended Fischer's lock and key metaphor to suggest that an enzyme's active site is complementary to the transition state for the reaction that it catalyses. The concept has been further developed, primarily by Wolfenden,[1,107] and a wide range of inhibitors that are analogues of a presumed transition state, or perhaps more closely, of a probable reactive intermediate has been described. The parallels between inhibitor and reactive intermediate structure make satisfying organic chemistry.

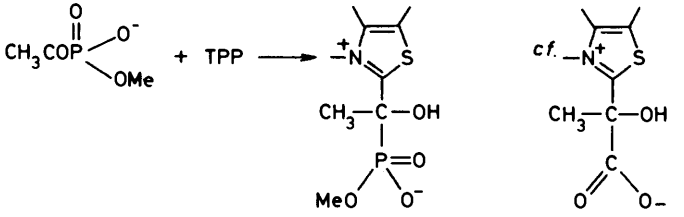
To revert to a reaction discussed earlier, pyruvate decarboxylase is inhibited by a phosphonate analogue of pyruvate (Scheme 31a). Although the coenzyme, TPP, adds to the carbonyl group, the adduct so formed cannot, of course, undergo decarboxylation and it blocks the active site.[108] The use of a phosphonate, which is a stable tetrahedral anionic group, to imitate a transient tetrahedral intermediate of an acyl group hydrolysis has also been a productive device. For example, a phosphonate analogue of a typical dipeptide substrate of carboxypeptidase is a potent inhibitor (Scheme 31b).[109] In this case, it is believed that the structure of the inhibitor matches the negatively charged phosphonate accurately, allowing bonding to the zinc ion at the enzyme's active site. The shape of a molecule alone can also provide a sufficiently close relationship to the binding preferences of an enzyme to cause inhibition. Thus, in racemization of a chiral centre, at some stage in the reaction a planar configuration is likely to be reached. An early example of a transition-state analogue showed this feature; the racemization of proline was inhibited by pyrrole-2-carboxylic acid; a planar analogue of the substrate[110] (Scheme 31c).

A great merit of the transition-state analogue concept is that it offers a rational approach to the design of potent specific inhibitors of enzymes from mechanistic information or even from speculation.[1,91] The relationship between inhibitors designed on the basis of transition-state structure is, of course, very close to the design of suicide inhibitors mentioned above. In the former case, the inhibitor is a molecule closely related to a structure on the normal reaction path: in the latter, the enzyme's normal mechanism is followed only part of the way until a branch that leads to destruction is reached.

Finally, moving to the very earliest moments of the transformation of a substrate molecule bound at an enzyme's active site, there has been much discussion over the years about whether an enzyme distorts its substrate on binding. Undoubtedly

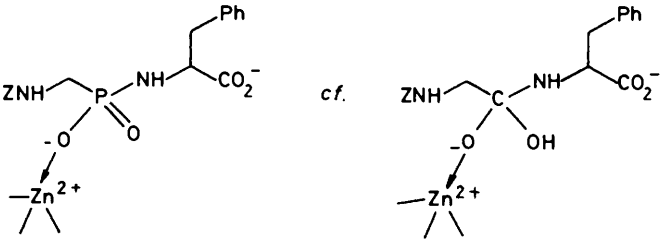
[106] L. Pauling, *Nature*, 1948, **161**, 707.
[107] R. Wolfenden, *Acc. Chem. Res.*, 1972, **5**, 10.
[108] R. Kluger and D. C. Pike, *J. Am. Chem. Soc.*, 1979, **101**, 6425.
[109] N. E. Jacobsen and P. A. Bartlett, *J. Am. Chem. Soc.*, 1981, **103**, 654.
[110] G. J. Cardinale and R. H. Abeles, *Biochemistry*, 1968, **7**, 3970.

(a) Pyruvate decarboxylase inhibition



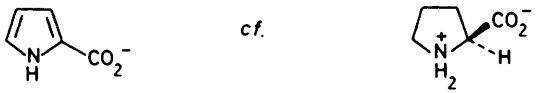
inhibitor intermediate

(b) Carboxypeptidase inhibition



inhibitor intermediate

(c) Proline racemase inhibition



inhibitor substrate

Scheme 31

both enzyme and substrate molecules have some flexibility and it would be surprising if mutual adjustments were not made on binding. If a substrate undergoes such a distortion along the reaction co-ordinate, then distortion on binding may contribute to catalysis, as has been argued for lysozyme. Recently, using spectroscopic techniques, it has been possible to detect enzyme–substrate interactions in solution before the chemical transformation takes place. For example, strong evidence for the Lewis acid activity of the zinc ion in horse liver alcohol dehydrogenase has come from a resonance Raman spectroscopic study of the binding of 4-dimethylaminobenzaldehyde to the enzyme.[111] This compound has a carbonyl stretching frequency at 1664 cm^{-1} that is lost on binding to the

[111] P. W. Jagodzinski and W. L. Peticolas, *J. Am. Chem. Soc.*, 1981, **103**, 234.

enzyme and is similarly lost by the model reaction of complexing with zinc ions in an organic solvent. The result was interpreted as evidence for the polarization of the carbonyl group of the substrate by zinc prior to hydride transfer from NADH.[51–53,111] Fourier transform infrared spectroscopy has detected a 19 cm^{-1} shift to lower frequency in the carbonyl absorption of dihydroxyacetone phosphate bound to triose phosphate isomerase.[112] Knowles has argued that this shift is due to a distortion of the carbonyl C—C—C bond angle by 9° towards a single bond, as required in the reaction product glyceraldehyde 3-phosphate.

6 Conclusion

The foregoing discussion has concentrated upon only one aspect of catalysis by enzymes, that relating to reactive intermediates. The convenience in discussion permitted by this limitation, however, should not be allowed to obscure the contribution of the whole enzyme molecule to catalysis in its natural environment. A minimum requirement in this regard is that the whole enzyme molecule must be soluble in its working environment, which may be aqueous, as in intracellular solution, or hydrophobic, as in lipid membranes.[113] The stabilization of reactive intermediates by enzymes and coenzymes is, of course, advantageous for catalysis but its effect is simply to transfer the onus of the rate-limiting step from bond making and breaking to the association and dissociation of reactants from the enzyme's active site. The ultimate biological catalyst, as Knowles and Albery first pointed out,[114] is an enzyme in which all the chemical steps proceed at optimal rates and hence diffusion becomes rate-limiting. When this situation has been reached, the only way in which an organism can improve the efficiency of its cnemistry is to bring together enzymes required for a particular biosynthetic pathway into an organized multi-enzyme complex.[113] The construction of such a system clearly depends upon intermolecular interactions between enzyme molecules. As has been described above, although uncertainties remain, much is known about the chemistry of the insides of enzymes, the active sites; much remains to be discovered about the chemistry of their outsides.

[112] J. G. Belasco and J. R. Knowles, *Biochemistry*, 1980, **19**, 472.
[113] N. C. Price and L. Stevens 'Fundamentals of Enzymology', Oxford University Press, Oxford. 1982.
[114] J. R. Knowles and W. J. Albery, *Acc. Chem. Res.*, 1977, **10**, 105.