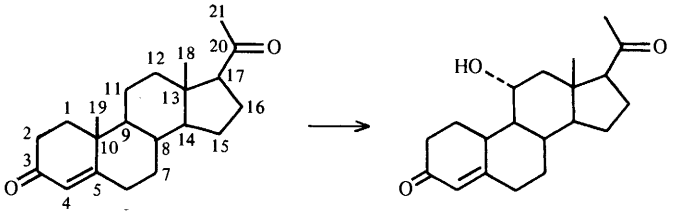
The Mechanism of the Microbial Hydroxylation of Steroids

By H. L. Holland
DEPARTMENT OF CHEMISTRY, BROCK UNIVERSITY, ST. CATHARINES, ONTARIO, CANADA, L2S 3A1

1 Introduction

The use of a micro-organism to introduce the hydroxy-group into an organic molecule by performing the direct conversion of carbon–hydrogen bond to carbon–hydroxyl with defined regio- and stereo-specificity has given the organic chemist the ability to produce a wide range of compounds which may otherwise severely tax his or her synthetic ingenuity and skill. The first application of this technique was for the synthesis of the anti-inflammatory corticosteroids, and followed the discovery in 1950 by Murray and Peterson that the fungus *Rhizopus arrhizus* (isolated from the air in Kalamazoo, Michigan) was able to convert progesterone (1) into its 11α-hydroxy derivative (2) in high isolable yield (Scheme 1).[1,2]



(1) (2)

Scheme 1 *The C-11α hydroxylation of progesterone*[1]

Since that time, microbial hydroxylation has been extensively applied in the production of steroids on a commercial scale,[3] and has also been used to a lesser extent in the research-scale preparation of non-steroid organic molecules.[4] In the steroid field, microbial transformations, of which the hydroxylation reaction is but one example, now constitute a formidable synthetic arsenal.[5] The key position of the microbial hydroxylation reaction in this arsenal is assured by the relative inaccessibility of the products by conventional chemical

[1] H. C. Murray and D. H. Peterson, US Patent 2 602 769 (July 8, 1952).
[2] G. G. Hazen, *J. Chem. Educ.*, 1980, **57**, 291.
[3] G. Nomine, *Bull. Soc. Chim. Fr.*, 1980, II-18.
[4] K. Kieslich, 'Microbial Transformations of Non-steroid Cyclic Compounds', 1976, Georg Thieme, Berlin.
[5] K. Kieslich, *Bull. Soc. Chim. Fr.*, 1980, II-9.

means, and is reflected in the coverage given to this aspect of the subject in several reviews devoted to microbial transformations of steroids.[6-9]

In spite of the widespread application of microbial steroid hydroxylation in both industry and research over three decades, it is only in recent years that the mechanism of this reaction has been even partially understood. At the present time, our knowledge of the biochemistry of the process is more complete than our understanding of the chemistry concerned; this review will discuss both chemical and biochemical data relevant to the mechanism of the microbial steroid hydroxylation reaction.

2 The Reactants

A. The Enzymes Involved.—The term microbial hydroxylation has been loosely used to cover transformations carried out by either bacteria or fungi. Of these, the latter group of micro-organisms has been more extensively exploited and is of greater synthetic utility in the steroid field.[6,7] However, the hydroxylation reaction may be performed by enzymes from a wide range of sources, including plant and animal tissue. The use of isolated enzymes or enzyme preparations for hydroxylation on a synthetic scale is not widespread; this is attributable to the difficulty of isolation and instability of the enzyme preparations concerned.[10] The vast majority of microbial steroid hydroxylations has therefore been performed using either actively growing or resting cultures of fungi.[6,7]

Irrespective of the source of the steroid hydroxylating enzyme, the available evidence suggests that it is an iron-containing cytochrome P-450 dependent species which functions with the stoicheiometry shown in equation 1.[7,11,12]

$$R{-}H + NADPH + O_2 \rightarrow R{-}OH + NADP^+ + {}^-OH \qquad (1)$$

These enzymes, which incorporate one molecule of molecular oxygen into the substrate, are classified as mono-oxygenases. The cytochrome P-450 dependent mono-oxygenases are widely distributed among almost all forms of life,[10] and exist in both soluble and membrane-bound forms. Much of the mechanistic work on this class of enzymes has been performed using soluble enzymes from bacterial sources, such as the camphor hydroxylase from *Pseudomonas putida*,[10,13] or on enzymes of mammalian origin, but the available evidence suggests that all cytochrome P-450 dependent mono-oxygenases function by a similar

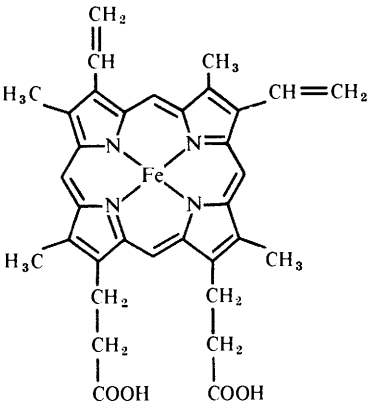
[6] W. Charney and H. L. Herzog, 'Microbial Transformations of Steroids', 1967, Academic Press, New York.
[7] L. L. Smith, in 'Terpenes and Steroids', (Specialist Periodical Reports), vol. 4, The Chemical Society, London, 1974, p. 394.
[8] C. Vezina and S. Rakhit in 'Handbook of Microbiology', vol. 4, ed. A. I. Laskin and H. A. Lechevalier, CRC Press, Cleveland, Ohio, 1974, p. 117.
[9] H. Iizuka and A. Naito, 'Microbial Transformations of Steroids and Alkaloids', University of Tokyo Press, Tokyo, and University Park Press, State College, Pennsylvania, 1967.
[10] 'Cytochrome P-450', ed. R. Sato and T. Omura, Academic Press, New York, 1978.
[11] K. Breskvar and T. Hudnik-Plevnik, *Biochem. Biophys. Res. Commun.*, 1977, **74**, 1192.
[12] K. Breskvar and T. Hudnik-Plevnik, *J. Steroid Biochem.*, 1981, **14**, 395.
[13] L. S. Alexander and H. M. Goff, *J. Chem. Educ.*, 1982, **59**, 179.

mechanism.[10] The data obtained from bacterial and mammalian enzymes are therefore relevant in a discussion of steroid hydroxylation mechanisms.

The existence of a multitude of hydroxylating enzymes which function with different substrate, regio-, and stereo-specificities, and yet are all dependent on the same cofactors, is now well established in mammalian systems.[14,15] The close similarity between hydroxylations performed by mammalian and fungal systems[16] suggests that a parallel state of affairs may exist in the microbial world.

The role of cofactors and polypeptide (apoenzyme) may be distinguished as follows: the cofactors are responsible for the binding of oxygen, its activation, and delivery to the substrate of the oxidizing species; and the apoenzyme is responsible for the binding and (if appropriate) activation of the organic substrate (steroid). The apoenzyme therefore controls the substrate, regio-, and stereo-specificity of the hydroxylation reaction, and it is variation in this portion of the enzyme which is largely responsible for the wide range of substrate specificities and products observed in this reaction. The role of the apoenzyme in interacting with the substrate is the least well understood aspect of the hydroxylation process. Since the interpretation of the mechanistic data relevant to this aspect of the process depends in part on a knowledge of the nature of the catalytic cycle, the latter will be discussed first.

B. The Binding and Activation of Oxygen.[10,17-19]—The overall features of the catalytic cycle of cytochrome P-450 mono-oxygenases appear to be independent

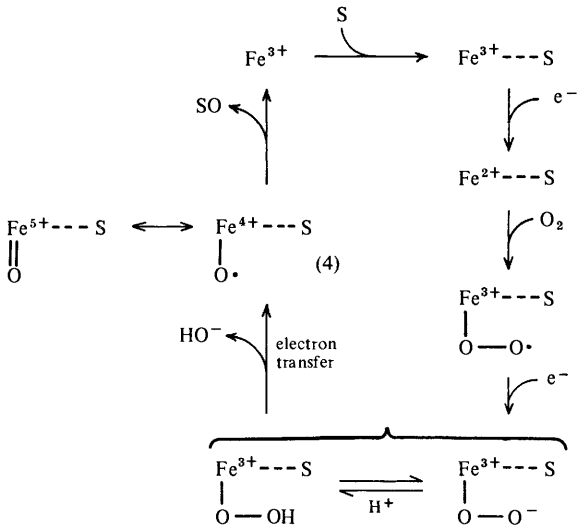


(3)

[14] M. A. Lang and D. W. Nebert, *J. Biol. Chem.*, 1981, **256**, 12058.
[15] M. A. Lang, J. E. Gielen, and D. W. Nebert, *J. Biol. Chem.*, 1981, **256**, 12068.
[16] R. V. Smith and J. P. Rosazza, *J. Pharm. Sci.*, 1975, **64**, 1737.
[17] 'Molecular Mechanisms of Oxygen Activation', ed. O. Hayaishi, Academic Press, New York, 1974.
[18] C. K. Chang and D. Dolphin in 'Bioorganic Chemistry', ed. E. E. van Tamelan, Academic Press, New York, 1978, vol. 4, p. 37.
[19] P. Bentley and F. Oesch in 'Foreign Compound Metabolism in Mammals', (Specialist Periodical Reports), vol. 5, The Chemical Society, London, 1979, p. 113.

of the source of the enzyme. The active site of cytochrome P-450$_{CAM}$ from *P. putida* contains an iron haem in the form of iron protoporphyrin IX (3), present in the resting state of the enzyme in the iron(III) state. The two axial ligands are provided by the protein; one of these is a cysteine sulphide ion, while the other, which is displaced by oxygen during the catalytic cycle, is currently unidentified but may be the imidazole nitrogen of a histidine residue.

The catalytic cycle of cytochrome P-450 dependent mono-oxygenases, which has been deduced largely from a study of the camphor hydroxylase of *P. putida*, is presented in Scheme 2. A detailed discussion of this cycle is beyond the



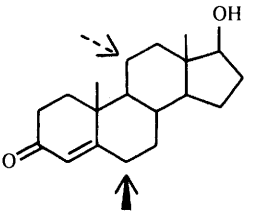
Scheme 2 *The catalytic cycle of cytochrome P-450 dependent mono-oxygenases (S = substrate)*

scope of this review, but the following points are relevant. The first step, substrate binding, is necessary before oxygen can bind to the iron centre; however, the subtrate does not bind directly to the haem unit, but is presumably bound by the apoprotein in close proximity to the cofactor. The two reducing equivalents are provided ultimately by NADPH, and are transferred to the cofactor *via* a flavin nucleotide, iron–sulphur proteins (ferredoxins) and/or cytochrome b_5, depending upon the source of the enzyme. The ultimate oxidizing species, here formulated as (4), has already lost one atom of molecular oxygen to the aqueous medium. The nature of (4) and its subsequent reaction to provide the product will be discussed in more detail in Section 3.

C. Binding of the Substrate.—The binding of substrate to a cytochrome P-450 dependent mono-oxygenase is accompanied by changes in both the conformation of the protein[20] and the spectral properties of the cofactor.[19] It has been

[20] H. Shichi, K. Kumaki, and D. W. Nebert. *Chem.-Biol. Interact.*, 1978, **20**, 133.

proposed that ketosteroids may bind to a protein as imines (*via* condensation with a primary amino-group)[21,22] or thio-ethers (*via* Michael addition of a thiol to Δ^4-3-ketosteroids).[23,24] However, hydroxylation of [3-^{18}O]testosterone (5) at C-6β or C-11α by *Rhizopus arrhizus* proceeded with *ca.* 80% retention of label,[25] a result which eliminates from consideration the binding of substrate as an imine with consequent loss of the original C-3 oxygen to the medium.

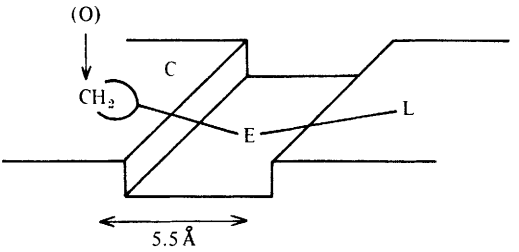


(5)

The binding of (5) as a thio-ether cannot be eliminated on this evidence, but is unlikely in view of the requirement of the C-6β hydroxylation for an intact Δ^4-3-ketosteroid substrate (*vide infra*). In the C-6β hydroxylation of (5), and the hydroxylation of related ketosteroids at positions adjacent to carbonyl, it is likely that binding of the substrate occurs in the enol form. This is discussed in greater detail in Section 2.D below.

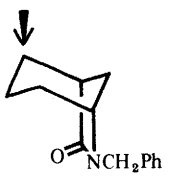
The first indication that a specific relationship may exist between the position of substitution of the substrate and the site of hydroxylation was provided by the work of Murray *et al.*[26,27] Using substituted cyclic and polycyclic substrates they postulated the existence in *Sporotrichum sulphurescens* of an enzyme–substrate complex in which oxygenation occurs at a methylene group about 5.5 Å away from an electron-rich substituent of the substrate (Figure 1). More recent work[28,29] with the same micro-organism (since reclassified as *Beauveria bassiana* and described[28] as *Beauveria sulphurescens*) using the bridged bicyclic and polycyclic amide substrates (6)—(12) has established the sites of mono-hydroxylation shown on the corresponding structures. Since these hydroxylation do not occur systematically 5.5 Å away from the carbonyl oxygen, the authors suggest that either the amide nitrogen or lipophilic aromatic ring rather than the carbonyl oxygen may be instrumental in determining the regiospecificity of hydroxylation for these substrates.

[21] W. F. Benisek and A. Jacobson, *Bio-org. Chem.*, 1975, **4**, 41.
[22] D. C. Wilton, *Biochem. J.*, 1976, **155**, 487.
[23] R. C. Fahey, P. A. Meyers, and D. L. DiStefano, *Bio-org. Chem.*, 1980, **9**, 293.
[24] C.-C. Chin and J. C. Warren, *Biochemistry*, 1972, **11**, 2720.
[25] H. L. Holland and G. J. Taylor, *Can. J. Chem.*, 1980, **58**, 2326.
[26] G. S. Fonken, M. E. Herr, H. C. Murray, and L. M. Reineke, *J. Am. Chem. Soc.*, 1967, **89**, 672.
[27] R. A. Johnson, M. E. Herr, H. C. Murray, and G. S. Fonken, *J. Org. Chem.*, 1968, **33**, 3182.
[28] R. Furstoss, A. Archelas, B. Waegell, J. Le Petit, and L. Deveze, *Tetrahedron Lett.*, 1980, **21**, 451.
[29] R. Furstoss, A. Archelas, B. Waegell, J. Le Petit, and L. Deveze, *Tetrahedron Lett.*, 1981, **22**, 445.

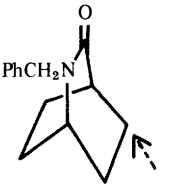


E = an electron-rich group
L = a lipophilic group which may or may not be part of C
C = a cyclic system

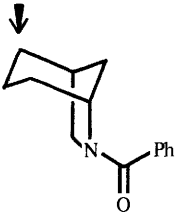
Figure 1 *Hypothetical enzyme–substrate complex for hydroxylation by* S. sulphurescens[26,27]



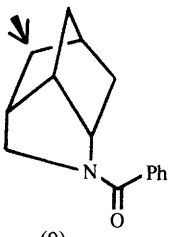
(6)



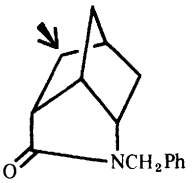
(7)



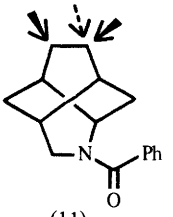
(8)



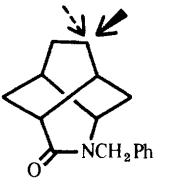
(9)



(10)



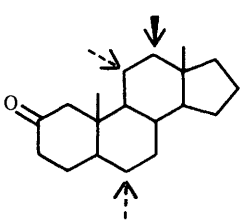
(11)



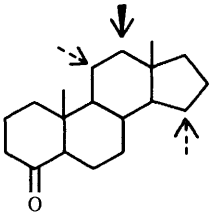
(12)

In the steroid field, this approach has been successfully applied over the past decade by the Oxford research group of Sir Ewart R. H. Jones and G. D. Meakins. A preliminary review of this work has appeared.[30] By employing steroid substrates with oxygen substituents (hydroxy and/or carbonyl) in defined locations, and varying these locations in a systematic manner, a relationship

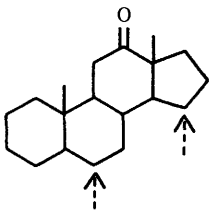
[30] E. R. H. Jones, *Pure Appl. Chem.*, 1973, **33**, 39.



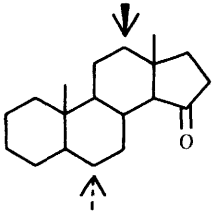
(13)



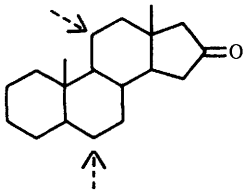
(15)



(16)



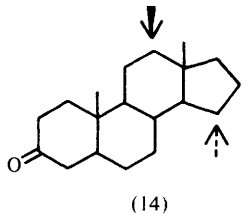
(17)



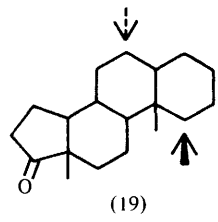
(18)

has been established between the position of substitution of the substrate and the site of hydroxylation of the latter for several micro-organisms.

Working with the mono-oxygenated 5α-androstane substrates (13)—(19) and the fungus *Calonectria decora*, the pattern of dihydroxylation shown on the structures was established.[31] The major transformation products contained two equatorial hydroxy-groups about 4 Å apart, and the sites of hydroxylation bear the approximate geometrical relationship to the position of the carbonyl substituent illustrated in Figure 2. This relationship also holds for several A-nor and D-homosteroids.[31]



(14)



(19)

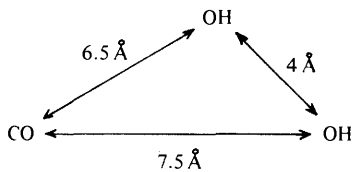
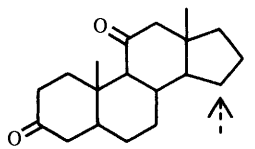


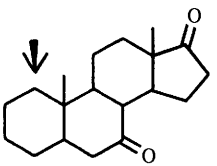
Figure 2 *Dihydroxylation of monoketo 5α-androstanes by* C. decora[31]

[31] A. M. Bell, P. C. Cherry, I. M. Clark, W. A. Denny, Sir Ewart R. H. Jones, G. D. Meakins. and P. D. Woodgate, *J. Chem. Soc., Perkin Trans. 1*, 1972, 2081.

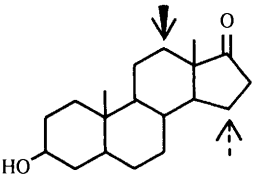
Further work[32] with di-oxygenated substrates and *C. decora* again resulted in equatorial hydroxylations, the predominant products being mono- or di-hydroxylated. The pattern of hydroxylation of the substrate diones or keto-alcohols was not so apparent as in the case of the monoketo substrates (14)—(19), but may be summarized as follows: The presence of a carbonyl or hydroxy-group in the A or D ring exerts a dominant directing influence which results in the introduction of hydroxyl according to the relationship of Figure 2 [*e.g.*, (20), (21)]. However, the presence of a carbonyl or hydroxy-group at a site close to a predicted position of hydroxylation appears to inhibit hydroxylation at that position [*e.g.*, C-6α, structure (20)], and substituents in the B or C ring do not show a strong directing influence. Nevertheless, with these constraints, the pattern of hydroxylation of disubstituted androstanes is roughly similar to that shown in Figure 2. The fact that at a given location, both carbonyl [*e.g.*, (14)] and hydroxyl [*e.g.*, (22)] exhibit similar directing effects supports the interpretation[32] of the enzyme–substrate binding as a hydrophilic interaction (*vide supra*). The sensitivity of the relationship shown in Figure 2 to changes in substrate geometry is reflected in the deviation from this pattern observed when 3α,5-cyclosteroids, *e.g.*, (23), *cf.* (19) were used as substrates.[33]



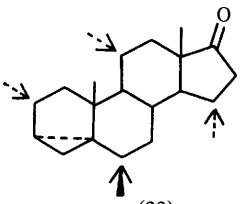
(20)



(21)



(22)

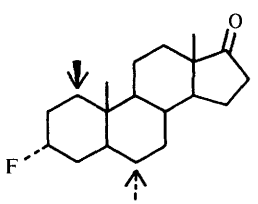


(23)

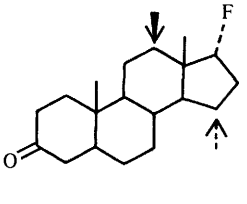
Oxygenated substituents other than carbonyl and hydroxyl, such as enol ethers and acetals, may also exert a directing influence on hydroxylation by *C. decora* similar to that of Figure 2, but generally with reduced yield and specificity.[34] The directing influence of halogen has also been studied using

[32] A. M. Bell, W. A. Denny, Sir Ewart R. H. Jones, G. D. Meakins, and W. E. Muller, *J. Chem. Soc., Perkin Trans. 1*, 1972, 2759.
[33] V. E. M. Chambers, W. A. Denny, Sir Ewart R. H. Jones, G. D. Meakins, J. O. Miners, J. T. Pinhey, and A. L. Wilkins, *J. Chem. Soc., Perkin Trans. 1*, 1975, 1359.
[34] J. M. Evans, Sir Ewart R. H. Jones, G. D. Meakins, J. O. Miners, A. Pendlebury, and A. L. Wilkins, *J. Chem. Soc., Perkin Trans. 1*, 1975, 1356.

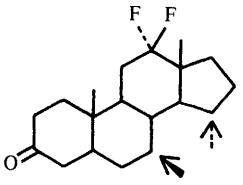
halosteroids as substrates for *C. decora*, *Rhizopus nigricans*, and *Aspergillus ochraceus*.[35-37] The position of hydroxylation of monoketohalosteroids by *C. decora* is controlled by the directing influence of the carbonyl group,[35] *e.g.*, (24), *cf.* (19), and (25), *cf.* (14), unless halogen is present at a preferred site of location, in which case hydroxylation occurs elsewhere, *e.g.*, (26), *cf.* (14).[36] With *R. nigricans* (*R. stolonifer*), hydroxylation may also occur at the preferred site irrespective of the presence of halogen [*e.g.*, (27), (28)],[35,37] whereas the position of hydroxylation by the C-11α site-specific hydroxylator *A. ochraceus* (*vide infra*) is unaffected by the presence of halogen [(29) and (30)] provided that the latter is not located close to C-11;[35,36] in this event, hydroxylation occurs elsewhere. With the exception, therefore, of hydroxylation by *R. nigricans* (*stolonifer*), the presence of a halogen substituent at C-*n* has little directing influence; it generally results in hydroxylation remote from that site when C-*n* is a favoured position of hydroxylation, and has little effect where the favoured hydroxylation site is remote from C-*n*.



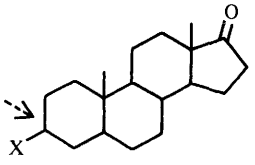
(24)



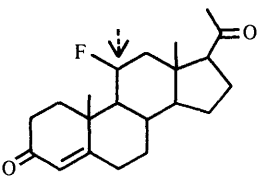
(25)



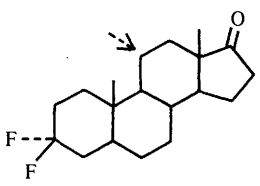
(26)



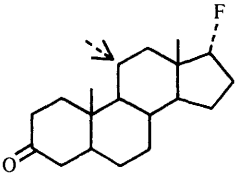
(27)



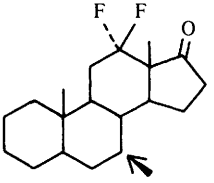
(28)



(29)



(30)

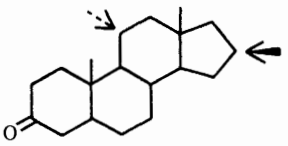


(31)

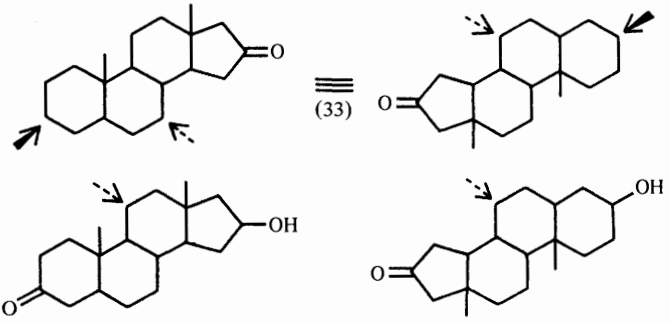
[35] Sir Ewart R. H. Jones, G. D. Meakins, J. O. Miners, and A. L. Wilkins, *J. Chem. Soc., Perkin Trans. 1*, 1975, 2308.

[36] T. G. C. Bird, P. M. Fredericks, Sir Ewart R. H. Jones, and G. D. Meakins, *J. Chem. Soc., Perkin Trans. 1*, 1980, 750.

[37] H. L. Holland and E. M. Thomas, *Can. J. Chem.*, 1982, **60**, 160.



(32)



(33)

(34) (35)

Several other fungi show a pattern of hydroxylation similar to the relationships deduced for *C. decora*. *Rhizopus nigricans*, although giving predominantly C-11α-hydroxylated products with pregnanes such as progesterone (1) (*cf.* Scheme 1),[38] exhibits a substituent directed pattern of hydroxylation with 5α-androstane substrates.[39,40] Using a series of monoketone, diketone, and keto-alcohol substrates, the relationship shown in Figure 3 was deduced, in which three sites exist on the enzyme which can fulfill either binding or hydroxylating roles. Monoketo substrates, *e.g.*, (32) and (33), are thus dihydroxylated following binding of the substrate carbonyl to one of these sites, whereas di-oxygenated substrates, *e.g.*, (34) and (35) are monohydroxylated at the third site following binding of the substrate to the other two. The possibility exists that the substrate may bind in either 'normal' [*e.g.*, (34)] and/or 'reverse' [*e.g.*, (35)] fashion.

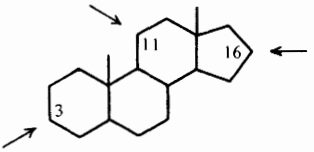
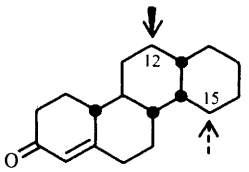


Figure 3 *Binding and hydroxylation of* 5α-*androstanes by* R. nigricans[39,40]

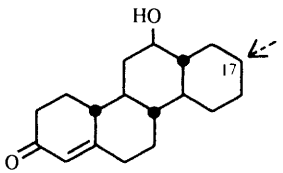
[38] D. H. Peterson and H. C. Murray, *J. Am. Chem. Soc.*, 1952, **74**, 1871.
[39] J. W. Browne, W. A. Denny, Sir Ewart R. H. Jones, G. D. Meakins, Y. Morisawa, A. Pendlebury, and J. Pragnell, *J. Chem. Soc., Perkin Trans. 1*, 1973, 1493.
[40] V. E. M. Chambers, W. A. Denny, J. M. Evans, Sir Ewart R. H. Jones, A. Kasal, G. D. Meakins, and J. Pragnell, *J. Chem. Soc., Perkin Trans. 1*, 1973, 1500.

The fungus *Rhizopus arrhizus* also shows a pattern of hydroxylation of 5α-androstanes similar to that of *R. nigricans*, but no clear relationship was discernable for hydroxylations performed by *Rhizopus circinnans*, apart from a tendency for hydroxylation in ring B or C.[41] A triangular geometry relating binding and hydroxylation of 5α-androstanes has been proposed for hydroxylation by *Wojnowicia graminis*,[42] *Ophiobolus herpotrichus*,[42] *Daedalea rufescens*,[43] *Diaporthe celastrina*,[44] and an unspecified fungus species.[45] In the case of *W. graminis* and *O. herpotrichus*,[42] the central site appears to have only binding, and not hydroxylating, capability, whereas with *D. rufescens*,[43] although all three sites have a dual function, the terminal ring sites are considered to be the primary hydroxylating entities; *D. celastrina*[44] exhibited a preference for α-face hydroxylation within the general framework of a triangular site arrangement. The unspecified fungus isolated from an ant nest ('Acromyrex fungus')[45] performed a variety of transformations in addition to hydroxylation, but the latter process could again be rationalized by the existence of a triangular arrangement of three enzyme sites, similar to that shown in Figure 3.

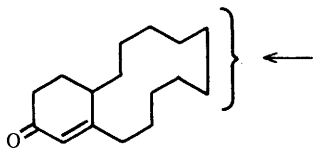
Site-directed hydroxylation has also been observed with several steroid-related substrates.[46] *C. decora* hydroxylated (36) as shown [*cf.* (14)], and *R. nigricans* hydroxylated (37) in the anticipated region of the molecule. Although bicyclic[47] substrates such as (38) were hydroxylated by *C. decora* and *R. nigricans* in the



(36)



(37)

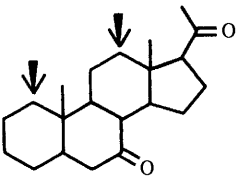


(38)

[41] A. M. Bell, I. M. Clark, W. A. Denny, Sir Ewart R. H. Jones, G. D. Meakins, W. E. Muller, and E. E. Richards, *J. Chem. Soc., Perkin Trans. 1*, 1973, 2131.
[42] V. E. M. Chambers, Sir Ewart R. H. Jones, G. D. Meakins, J. O. Miners, and A. L. Wilkins, *J. Chem. Soc., Perkin Trans. 1*, 1975, 55.
[43] A. M. Bell, Sir Ewart R. H. Jones, G. D. Meakins, J. O. Miners, and A. Pendlebury, *J. Chem. Soc., Perkin Trans. 1*, 1975, 357.
[44] A. M. Bell, A. D. Boul, Sir Ewart R. H. Jones, G. D. Meakins, J. O. Miners, and A. L. Wilkins, *J. Chem. Soc., Perkin Trans. 1*, 1975, 1364.
[45] Sir Ewart R. H. Jones, G. D. Meakins, J. O. Miners, J. H. Pragnell, and A. L. Wilkins, *J. Chem. Soc., Perkin Trans. 1*, 1975, 1552.
[46] M. J. Ashton, A. S. Bailey, and Sir Ewart R. H. Jones, *J. Chem. Soc., Perkin Trans. 1*, 1974, 1658.
[47] A. S. Bailey, M. L. Gilpin, and Sir Ewart R. H. Jones, *J. Chem. Soc., Perkin Trans. 1*, 1977, 265.

range 7—9 Å away from an existing oxygen substituent, the conversions and isolated yields were very low. The use of monocyclic substrates such as cyclododecanone and cyclopentadecanone did not yield useful mechanistic information beyond the generalization that initial hydroxylation occurred at a position remote from the directing carbonyl substituent.[48]

Although *C. decora*, *R. nigricans*, *R. arrhizus*, and *D. rufescens* exhibit the site directed hydroxylation of 5α-androstanes discussed above, these fungi gave complex mixtures and less regio-selective hydroxylation when metabolizing 5α-pregnane substrates.[49] However, general similarities were apparent, so that hydroxylation of a C-*n* oxygenated 20-ketopregnane usually occurred at the same position as that of a C-*n* oxygenated 17-ketoandrostane [*e.g.*, (39) with *C. decora cf.* (21)]. The difference in hydroxylating behaviour between the three *Rhizopus* species observed for androstane substrates (*vide supra*) was also present, but less markedly so, for pregnane substrates.



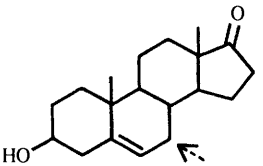
(39)

Fungi have also been identified which clearly do not have a definite geometrical relationship between substrate substituent(s) and site of hydroxylation. Thus *Absidia regnieri* and *Syncephalastrum racemosum*, although both active hydroxylators of 5α-androstane derivatives, do not exhibit a clear relationship between site of hydroxylation and substrate structure.[50] *Aspergillus ochraceus*, on the other hand, shows a predilection for hydroxylation at C-11α of a wide range of substrates in both the androstane[51] and pregnane[52] series, irrespective of the location of substitution in the substrate.

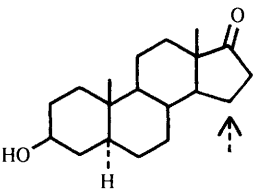
D. Activation of the Substrate.—In the hydroxylations at saturated carbon discussed above, (with the possible exception of those by *A. ochraceus*), it is assumed that no specific activation of the C—H bond concerned is provided by the enzyme, but rather the position of hydroxylation is controlled by the geometrical nature of the active site. However, not all steroid C—H bonds

[48] M. J. Ashton, A. S. Bailey, and Sir Ewart R. H. Jones, *J. Chem. Soc., Perkin Trans. 1*, 1974, 1665.
[49] Sir Ewart R. H. Jones, G. D. Meakins, T. O. Miners, R. N. Mirrington, and A. L. Wilkins, *J. Chem. Soc., Perkin Trans. 1*, 1976, 1842.
[50] A. M. Bell, Sir Ewart R. H. Jones, G. D. Meakins, J. O. Miners, and A. L. Wilkins, *J. Chem. Soc., Perkin Trans. 1*, 1975, 2040.
[51] A. M. Bell, J. W. Browne, W. A. Denny, Sir Ewart R. H. Jones, A. Kasal, and G. D. Meakins, *J. Chem. Soc., Perkin Trans. 1*, 1972, 2930.
[52] A. S. Clegg, W. A. Denny, Sir Ewart R. H. Jones, G. D. Meakins, and J. T. Pinhey, *J. Chem. Soc., Perkin Trans. 1*, 1973, 2137.

are equally reactive towards hydroxylation, and this phenomenon can also play a role in determining the position of microbial hydroxylation. For example, dry ozonation of saturated steroids supported on silica gel, reaction conditions reported to mimic microbial hydroxylation,[53] demonstrate selectivity in oxidation at C-14,[54] and hydroxylation at allylic positions of steroidal and related olefins is common.[55–57] Indeed, allylic hydroxylation becomes the dominant pathway of metabolism for the unsaturated substrate (40) whose saturated analogue (41) is hydroxylated (in this case by *Fusarium graminearum*) at a contiguous but different site.[58]

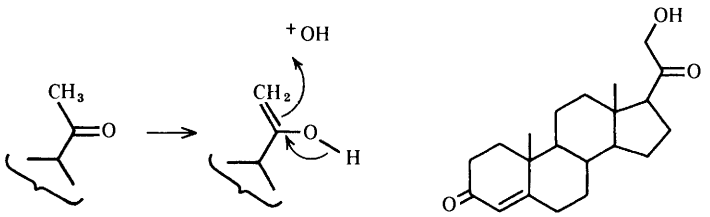


(40)



(41)

Although enzymic C—H bond activation at saturated non-allylic carbon is apparently unnecessary for hydroxylation, and indeed it is difficult to visualise how such activation could occur in an enzymic system, the situation is potentially more complex for hydroxylations which occur adjacent to carbonyl or conjugated carbonyl groups. During the hydroxylation of, for example, progesterone (1), at C-2, -6, -17 or -21, activation of carbon towards electrophilic oxidation can occur by enolization,[59] shown in Scheme 3 for hydroxylation at C-21. The oxidizing species is shown for mechanistic convenience as '$^{+}$OH'; its exact nature is discussed in Sections 2.B and 3.A. Circumstantial evidence for the involvement of enolic intermediates has existed for some time; thus

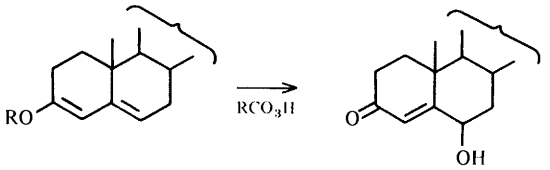


(1) (42)

Scheme 3 *Activation of* C-21 *towards electrophilic attack by enolization*

[53] A. L. J. Beckwith and T. Duong, *J. Chem. Soc., Chem. Commun.*, 1978, 413.
[54] R. L. Wife, D. Kyle, L. J. Mulheirn, and H. C. Volger, *J. Chem. Soc., Chem. Commun.*, 1982, 306.
[55] T. A. Crabb, P. J. Dawson, and R. O. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1980, 2535.
[56] T. A. Crabb, P. J. Dawson, and R. O. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1982, 571.
[57] R. A. LeMahieu, B. Tabenkin, J. Berger, and R. W. Kierstead, *J. Org. Chem.*, 1970, **35**, 1687.
[58] G. Defaye, M. H. Luche, and E. M. Chambaz, *J. Steroid Biochem.*, 1978, **9**, 331.
[59] H. J. Ringold, in 'Oxygenases', ed. D. Hayaishi, Academic Press, New York, 1962, p. 227.

hydroxylations at the axial C-2β, -6β, and -10β (in 19-norsteroids) positions of Δ^4-3-ketosteroids are among the most frequently reported, whereas hydroxylations at C-2α, -6α, and -10α are quite rare.[6,7] Chemical electrophilic attack on the appropriate enolic species leads to preferential axial substitution under stereoelectronic control,[60,61] exemplified by the peracid oxidation of $\Delta^{3,5}$-dienol derivatives to give exclusively 6β-hydroxy-Δ^4-3-ketones, shown in Scheme 4.[62] The facile electrophilic oxidation of steroidal enols,[62-65] and the existence of enzymes which enolize ketosteroids,[66] add credence to this mechanistic proposal.

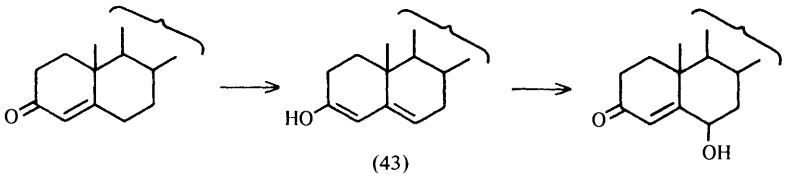


Scheme 4 *Peracid oxidation of $\Delta^{3,5}$-dienol derivatives*

The possibility of enolization during the C-21 hydroxylation of progesterone (1) by *Aspergillus niger* has been examined using substrates with one, two, and three deuterium atoms at C-21.[67] Using [21-$^2H_3$]progesterone as substrate, the product (42) was obtained with two deuterium atoms at C-21. Hydroxylation therefore occurred without reversible enolization of the C-20 carbonyl towards C-21 with concomitant loss of label. However, prolonged incubation times can lead to subsequent non-enzymic exchange of label at C-21 of (42) with protium of the medium; the medium pH drops as low as 2.3 in actively growing cultures.[68] Substrates with one and two deuterium atoms at C-21 were used to obtain the primary intramolecular isotope effect for C-21 hydroxylation.[67] The value obtained, $k_H/k_D = 1.25$, is inconsistent with a mechanism requiring prior enolization (Scheme 3), and suggests that C-21 hydroxylation occurs by direct reaction of the oxidizing species with a C-21—H bond.

The microbial C-6β hydroxylation of Δ^4-3-ketosteroids by *Rhizopus arrhizus* has been shown to proceed *via* binding of the substrate to the enzyme as the $\Delta^{3,5}$-dienol (43) (Scheme 5).[69-74] The earlier report[75] that this reaction

[60] J. Romo, G. Rosenkranz, C. Djerassi, and F. Sondheimer, *J. Org. Chem.*, 1954, **19**, 1509.
[61] P. B. D. de la Mare and B. N. B. Hannan, *J. Chem. Soc., Perkin Trans. 2*, 1973, 1086.
[62] D. N. Kirk and J. M. Wiles, *J. Chem. Soc., Chem. Commun.*, 1970, 518, 1015.
[63] E. J. Bailey, J. Elks, and D. H. R. Barton, *Proc. Chem. Soc.*, 1960, 214.
[64] P. B. D. de la Mare and R. D. Wilson, *J. Chem. Soc., Perkin Trans. 2*, 1977, 157.
[65] H. L. Holland, E. Riemland, and U. Daum, *Can. J. Chem.*, in press.
[66] S. K. Malhotra and H. J. Ringold, *J. Am. Chem. Soc.*, 1965, **87**, 3228.
[67] H. L. Holland and B. J. Auret, *Can. J. Chem.*, 1975, **53**, 845.
[68] H. L. Holland and Jahangir, unpublished data.
[69] H. L. Holland and B. J. Auret, *Can. J. Chem.*, 1975, **53**, 2041.
[70] H. L. Holland and B. J. Auret, *Tetrahedron Lett.*, 1975, 3787.
[71] H. L. Holland and P. R. P. Diakow, *Can. J. Chem.*, 1978, **56**, 694.
[72] H. L. Holland and P. R. P. Diakow, *Can. J. Chem.*, 1979, **57**, 436.
[73] H. L. Holland and P. R. P. Diakow, *Can. J. Chem.*, 1979, **57**, 1585.
[74] H. L. Holland, *Can. J. Chem.*, 1981, **59**, 1651.
[75] S. Baba, H. J. Brodie, M. Hayano, D. H. Peterson, and O. K. Sebek, *Steroids*, 1963, 1, 151.

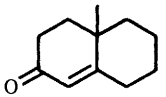


(43)

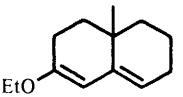
Scheme 5 C-6β *hydroxylation of* Δ^4-3-*ketosteroids by* **R.** arrhizus

preceeded with retention of tritium label at C-6α was originally interpreted as militating against the involvement of the dienol (43), but this work preceeded the investigations of Ringold and co-workers[66,76-78] on the stereoelectronic effects operative in the enolization of Δ^4-3-ketosteroids. Since enolization occurs with almost complete retention of the C-6α hydrogen, retention of label at C-6α during C-6β hydroxylation is not inconsistent with an enolic intermediate.

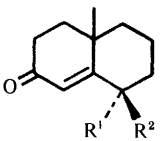
The first indication that an dienolic intermediate was involved came from incubation of the Δ^4-3-ketosteroid analogue (44) with *R. arrhizus*, and from peracid oxidation of the dienol-ether (45).[69] Both procedures yielded a mixture of the alcohols (46) and (47) in which (46) was predominant; the formation



(44)



(45)

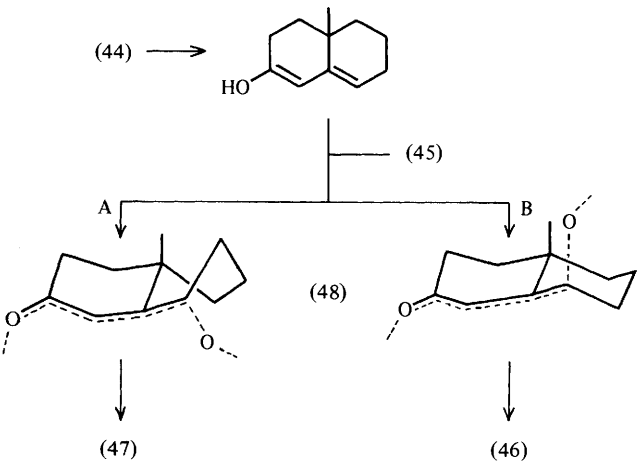


(46) R^1 = H, R^2 = OH

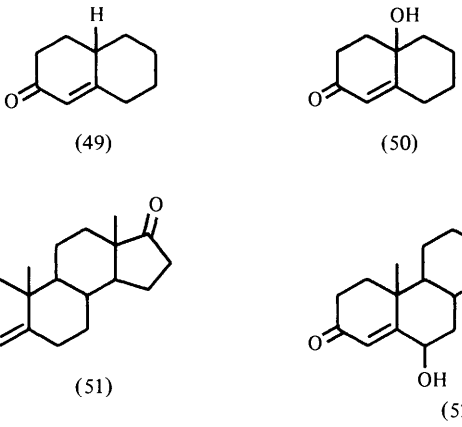
(47) R^1 = OH, R^2 = H

of this mixture was rationalized by the proposal that both enzymic and chemical oxidation proceeded by stereoelectronically controlled axial addition to the dienols (48) (Scheme 6). For the conformationally constrained steroid, only route B of Scheme 6 is feasible, and so only β oriented products are obtained. A similar result was obtained using the 19-nor steroid analogue (49).[71] The products in this case included the alcohol (50). Hydroxylation at C-10 is also observed with 19-nor-Δ^4-3-ketosteroid substrates,[71,79] presumably *via* $\Delta^{3,5(10)}$-dienol intermediates. The use of androst-4-ene-3,17-dione (51) with

[76] S. K. Malhotra and H. J. Ringold, *J. Am. Chem. Soc.*, 1963, **85**, 1538.
[77] S. K. Malhotra and H. J. Ringold, *J. Am. Chem. Soc.*, 1964, **86**, 1997.
[78] G. Subrahmanyam, S. K. Malhotra, and H. J. Ringold, *J. Am. Chem. Soc.*, 1966, **88**, 1332.
[79] J. Favero, J. Marchand, and F. Winternitz, *Bull. Soc. Chim. Fr.*, 1977, 310.

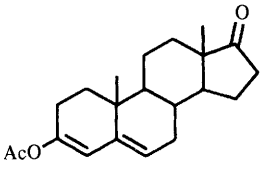


Scheme 6 *Hydroxylation by axial addition to a dienol*



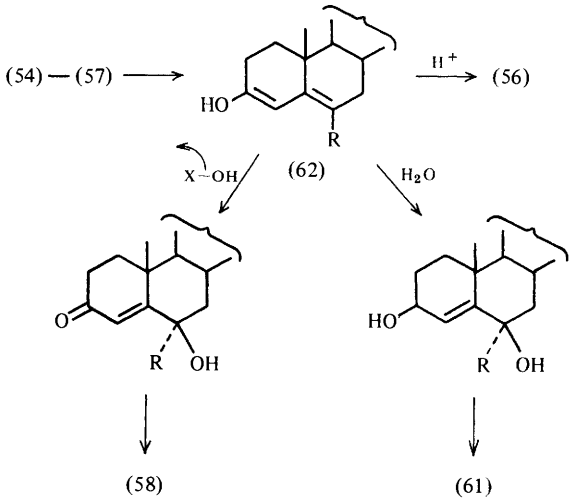
deuterium labels at C-4, C-6α, and C-6β as substrates for C-6β hydroxylation by *R. arrhizus* confirmed the intermediacy of (43) in the C-6β hydroxylation reaction.[71] The reaction proceeded with complete retention of label at C-4 and C-6α. The C-6β labelled substrate retained 12—15% of the original label, which was located at C-6α in the product (52). This result is in agreement with the observed retention of 17% label during enolization of C-6β deuterium labelled Δ^4-3-ketosteroids towards C-6,[78] and inconsistent with a direct stereospecific reaction of the oxidizing species at C-6β.

Further evidence for the pathway shown in Scheme 5 was provided by metabolism of the dienol acetate (53) by *R. arrhizus*.[70,71] Co-incubation of (53), and the ketone (51) labelled with deuterium at C-16, indicated that (53) was

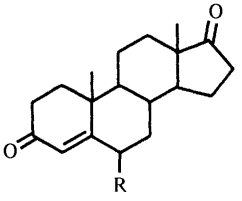


(53)

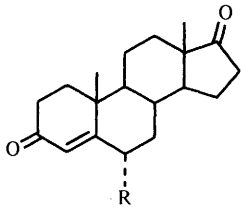
transformed to the alcohol (52) faster than was (51). The metabolism of C-6 substituted Δ^4-3-ketosteroids has also been examined, and the results rationalized in terms of the pathway of Scheme 7.[73] The C-6β halo-substituted substrates (54) and (55) gave products [(56), (58), and (61)], whose formation could be rationalized by the presence of the dienol (62) and the subsequent reactions shown in Scheme 7. Similar products were obtained upon incubation of the dienol acetate (59) with *R. arrhizus*, and upon peracid oxidation of the corresponding dienol ether (60).



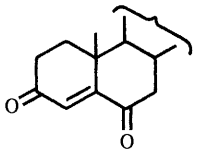
Scheme 7 *Metabolism of* C-6 *substituted* Δ^4-3-*ketosteroids by* **R.** arrhizus



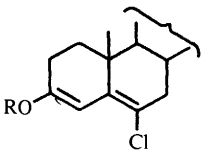
(54) R = Cl
(55) R = F



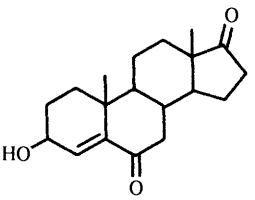
(56) R = Cl
(57) R = F



(58)

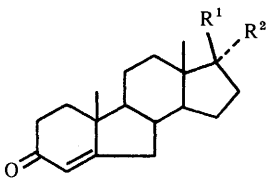


(59) R = Ac
(60) R = Et

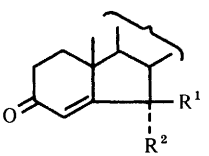


(61)

The assumption that the axial (β) stereochemistry of hydroxylation at C-6 in substrates such as (1), (5), and (51) is the product of stereoelectronic addition of oxygen to the corresponding dienol (43) is supported by results obtained from the incubations of the B-norsteroids (63) and (64) with *R. arrhizus*, when both α and β alcohols (65) and (66) were formed.[74] The conformation of the B ring in (63) and (64) is such that both α and β positions at C-6 are stereochemically equivalent with respect to the plane of the O—C-3—C-4—C-5—enone system, so that interaction of developing electron density at C-6 from a $\Delta^{3,5}$-dienol of (63) or (64) will occur equally favourably from both the α and β faces,



(63) $R^1 + R^2 = O$
(64) $R^1 = OH$, $R^2 = H$

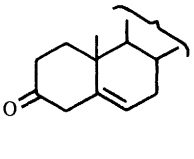


(65) $R^1 = H$, $R^2 = OH$
(66) $R^1 = OH$, $R^2 = H$

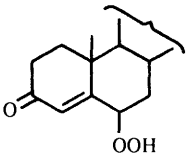
thus giving both (65) and (66) as products. The possibility that C-6β hydroxylation of Δ^4-3-ketosteroids proceeds *via* the Δ^5 isomer (67) and the hydroperoxide (68)[80] has also been examined.[72] Incubation of (67) with *R. arrhizus* gave a mixture of products which included (52) and (58), and which was apparently formed by auto-oxidation of the substrate. There is no direct evidence for the involvement of free (67) or (68) in enzymic C-6β hydroxylation.

Brodie *et al.* have studied the C-6β and C-7β hydroxylation of deuterium labelled estr-4-ene-3,17-dione (69), using *Botryodiplodia malorum*.[81,82] The interpretation of their results is difficult because of lack of homogeneity of label in the substrates, particularly at C-6β,[71,83] but nevertheless their data for C-6β hydroxylation are not inconsistent with the existence of an enolic intermediate and the route shown in Scheme 5. Hydroxylation at C-7β, however, occurs

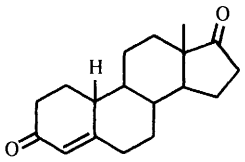
[80] P. H. Yu and L. Tan, *J. Steroid Biochem.*, 1977, **8**, 825.
[81] H. J. Brodie and C. E. Hay, *Biochem. J.*, 1970, **120**, 667.
[82] I. Kim, C. E. Hay, and H. J. Brodie, *J. Biol. Chem.*, 1973, **248**, 2134.
[83] H. L. Holland and G. J. Taylor, *Can. J. Chem.*, 1981, **59**, 2809.



(67)



(68)

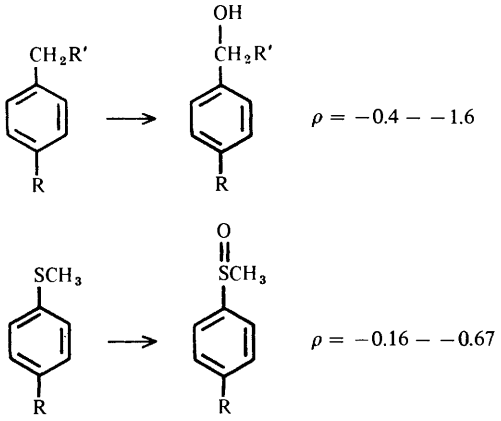


(69)

without loss of label from C-6β, and therefore cannot involve binding of the substrate as (43) unless formation of the latter is reversible and stereoselective, with return of the C-6β deuteron from the enzyme at the end of the reaction.

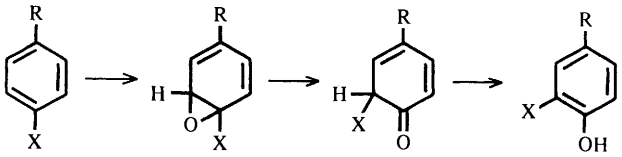
3 The Hydroxylation Reaction

A. The Nature of the Oxidizing Species.—The oxidizing species has been formulated as (4), Scheme 2.[84] Its electrophilic character is apparent from the observation that benzylic hydroxylation, and the closely related oxidation of alkyl aryl sulphides to sulphoxides (Scheme 8), a reaction also carried out by



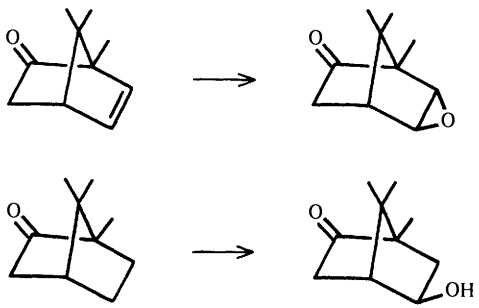
Scheme 8 *Hammett ρ values for benzylic hydroxylation and sulphoxidation*

[84] J. T. Groves, S. Krishnan, G. E. Avaria, and T. E. Nemo, in 'Biomimetic Chemistry', ed. D. Dolphin, C. McKenna, V. Murakami, and I. Tabushi, American Chemical Society, Washington, D. C., 1980, p. 277.



Scheme 9 *Formation of arene oxides during aromatic hydroxylation*

cytochrome P-450 dependent mono-oxygenases,[85] both proceed with rates which indicated a negative ρ value in the Hammett relationship.[86] The electrophilic nature of the oxidation is also indicated by studies using olefinic substrates. Hydroxylation of aromatic substrates can proceed *via* arene oxide intermediates and the well-known NIH shift (Scheme 9),[87,88] while other cytochrome P-450 dependent mono-oxygenases epoxidize dehydrosubstrates related to their normal saturated substrate at a rate similar to that of hydroxylation, both transformations occurring at the same enzyme site.[89] This is the case for the camphor hydroxylase of *P. putida* (Scheme 10),[90] and was first established for steroids with the C-9α hydroxylase from *Nocardia restrictus*.[91]



Scheme 10 *Epoxidation and hydroxylation by camphor hydroxylase*[90]

A stereochemical relationship between steroid hydroxylation and epoxidation was deduced by Bloom and Shull, who formulated the proposal that 'a micro-organism capable of introducing an axial hydroxy-function at C-*n* of a saturated steroid will also effect the introduction of an epoxide grouping axial at C-*n* in the corresponding unsaturated steroid'.[92] Equatorial hydroxylases do

[85] Y. Watanabe, T. Iyanagi, and S. Oae, *Tetrahedron Lett.*, 1982, **23**, 533.
[86] H. L. Holland and I. M. Carter, *Can. J. Chem.*, in press.
[87] R. A. Sheldon and J. K. Kochi, 'Metal-catalysed Oxidations of Organic Compounds', 1981, Academic Press, New York, p. 248.
[88] D. R. Boyd, R. M. Campbell, H. C. Craig, C. G. Watson, J. W. Daly, and D. M. Jerina, *J. Chem. Soc., Perkin Trans. 1*, 1976, 2438.
[89] R. T. Ruettinger and A. J. Fulco, *J. Biol. Chem.*, 1981, **256**, 5728.
[90] M. H. Gelb, P. Malkonen, and S. G. Sligar, *Biochem. Biophys. Res. Commun.*, 1982, **104**, 853.
[91] F. N. Chang and C. J. Sih, *Biochemistry*, 1964, **3**, 1551.
[92] B. M. Bloom and G. M. Shull, *J. Am. Chem. Soc.*, 1955, **77**, 5767.

not effect similar conversions. This epoxidation may occur because of the spatial resemblance of the π electron distribution in an unsaturated substrate to the area of maximum electron density in the related axial C—H bond of the corresponding saturated compound, shown for a C-11β hydroxylase in Figure 4.

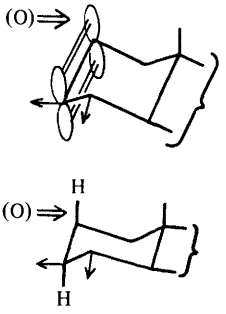
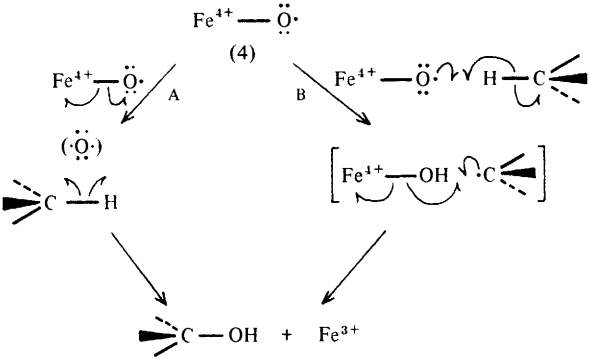


Figure 4 C-11β *hydroxylation and* C-9(11) *epoxidation*

B. The Stereochemistry of Hydroxylation.—Hydroxylation of steroids at unactivated positions occurs exclusively with net retention of configuration. This was first established for C-11α hydroxylation of C-11 labelled progesterones (*cf.* Scheme 1) by *R. nigricans*[93,94] and has since been found to be the case for all cytochrome P-450 dependent steroid hydroxylations which have been studied.[75] With the exception of a minority of cases, hydroxylation of other substrates also occurs with retention, and is considered the 'normal' mode of reaction.[95] An isolated report of hydroxylation with net inversion of configuration concerns the alkaloid norpluviine;[96] however, hydroxylation of related alkaloids occurred with retention or partial retention of configuration,[97] so that the observed inversion during norpluviine hydroxylation may be attributable to a combination of a stepwise radical hydroxylation mechanism and a large primary kinetic isotope effect (*vide infra*). Hydroxylations which occur with loss of configuration are a source of useful mechanistic data, and are considered below.

C. Interaction between the Substrate and the Oxidizing Species.—The oxidizing species (4) may react with substrate in two ways (paths A and B, Scheme 11). The observed retention of configuration (Section 3.B) and the ability of (4) to epoxidize unsaturated substrates (Section 3.A) led to the proposal that hydroxylation occurs by direct insertion of the six-electron oxene species into a C—H bond (path A).[98] The oxene mechanism of hydroxylation was accepted for many years as being the most consistent with the available

[93] E. J. Corey, G. A. Gregoriou, and D. H. Peterson, *J. Am. Chem. Soc.*, 1958, **80**, 2338.
[94] M. Hayano, M. Gut, R. I. Dorfmann, O. K. Sebek, and D. H. Peterson, *J. Am. Chem. Soc.*, 1958, **80**, 2336.
[95] J. T. Groves, *Adv. Inorg. Biochem.*, 1979, **1**, 119.
[96] I. T. Bruce and G. W. Kirby, *J. Chem. Soc., Chem. Commun.*, 1968, 207.
[97] C. Fuganti and M. Mazza, *J. Chem. Soc., Chem. Commun.*, 1972, 936.
[98] G. A. Hamilton, *J. Am. Chem. Soc.*, 1964, **86**, 3391.



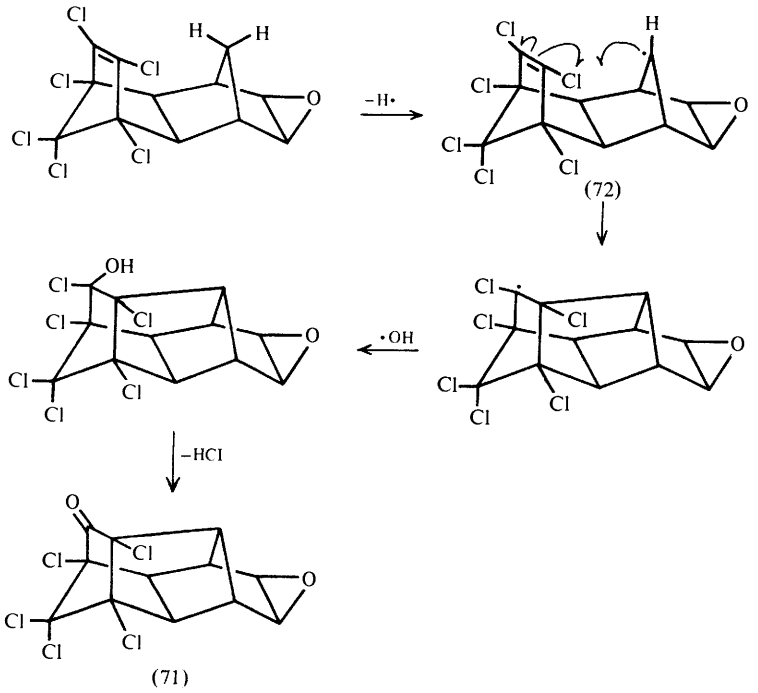
Scheme 11 *Possible routes for hydroxylation at saturated carbon*

experimental data, but recently the free radical mechanism (route B), proposed by Wiberg[99] for analogous chemical oxidations, has gained some acceptance.

(i) *Product and Configuration Studies.* The first positive indication that hydroxylation may proceed *via* radical intermediates came from a study of the metabolism of the insecticide dieldrin (70) in mammals.[100] The formation of (among others) the bridged metabolite (71) has been rationalized by the mechanism of Scheme 12,[101] involving a transannular reaction of the radical intermediate (72). Further evidence for radical intermediates has been provided by the hydroxylation of deuterium labelled norbornanes by a rabbit liver cytochrome P-450 system, which can occur with partial epimerization of label at the oxidized carbon,[102] and by the observation that the cumene hydroperoxide dependent hydroxylation of labelled cyclohexene (73) by a liver microsomal system proceeded with partial allylic rearrangement.[103] The production of 5-*exo*-hydroxycamphor from camphor by the camphor hydroxylase of *P. putida* (Scheme 10) also occurs with partial epimerization at C-5, and a radical intermediate has been proposed.[104]

The production of hydroxy-radicals by cytochrome P-450 dependent monooxygenase preparations has been inferred in several instances,[105–108] and the

[99] K. B. Wiberg, in 'Oxidation in Organic Chemistry', ed. K. B. Wiberg, Academic Press, New York, 1965, p. 69.
[100] M. K. Baldwin, J. Robinson, and D. B. Parke, *Food Cosmetics Toxicol.*, 1972, **10**, 333.
[101] C. T. Bedford, in 'Foreign Compound Metabolism in Mammals', (Specialist Periodical Reports), vol. 4, The Chemical Society, London, 1975, p. 407.
[102] J. T. Groves, G. A. McClusky, R. E. White, and M. J. Coon, *Biochem. Biophys. Res. Commun.*, 1978, **81**, 154.
[103] J. T. Groves, O. F. Akinbote, and G. E. Avaria, in 'Microsomes, Drug Oxidations and Chemical Carcinogenesis', vol. 1, ed. M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gillette, and P. J. O'Brien, Academic Press, 1980, p. 253.
[104] M. H. Gelb, D. C. Heimbrook, P. Mälkönen, and S. G. Sligar, *Biochemistry*, 1982, **21**, 370.
[105] K. Ohnishi and C. S. Lieber, *Arch. Biochem. Biophys.*, 1978, **191**, 798.
[106] G. Cohen and A. I. Cederbaum, *Arch. Biochem. Biophys.*, 1980, **199**, 438.
[107] M. Ingelman-Sundberg and G. Elkström, *Biochem. Biophys. Res. Commun.*, 1982, **106**, 625.
[108] F. Hawco, L. Hulett, and P. J. O'Brien, in ref. 103, p. 419.

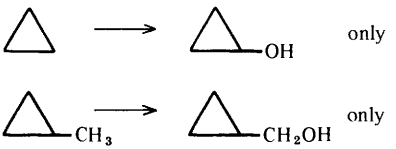


Scheme 12 *Rearrangement during dieldrin metabolism*[100,101]

existence of substrate based radicals during *N*-oxidation by similar systems has also been proposed.[109,110] The epoxidation of *trans,trans*-1,8-dideutero-1,7-octadiene with inversion of the original olefin geometry, using an enzyme of *Pseudomonas oleovorans*, has been also cited as supportive of a stepwise oxidation process analogous to radical hydroxylation,[111] but since the *P. oleovorans* enzyme does not require cytochrome P-450, the relevance of this finding to the mode of action of other mono-oxygenases is unclear. In experiments specifically designed to test for intermediacy of a substrate radical, Golding and co-workers examined the hydroxylation of cyclopropane and methylcyclopropane by *Methylococcus capsulatus* (Scheme 13).[112] No rearrangement products were detected, indicating that free charged or radical intermediates were not involved.

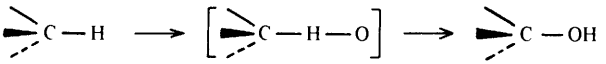
Theoretical calculations for the reaction of singlet carbene[113] and oxene[114] with a C—H bond indicate that the preferred pathway does not involve

[109] R. P. Hanzlik and R. H. Tullman, *J. Am. Chem. Soc.*, 1982, **104**, 2048.
[110] B. K. Sinha and A. G. Motten, *Biochem. Biophys. Res. Commun.*, 1982, **105**, 1044.
[111] S. W. May, S. L. Gordon, and M. S. Steltenkamp, *J. Am. Chem. Soc.*, 1977, **99**, 2017.
[112] H. Dalton, B. T. Golding, B. W. Waters, R. Higgins, and J. A. Taylor, *J. Chem. Soc., Chem. Commun.*, 1981, 482.
[113] R. C. Dobson, D. M. Hayes, and R. Hoffmann, *J. Am. Chem. Soc.*, 1971, **93**, 6188.
[114] A. T. Budzianowski and G. H. Loew, *J. Am. Chem. Soc.*, 1980, **102**, 5443.



Scheme 13 *Hydroxylation of cyclopropane and methylcyclopropane by* M. capsulatus[112]

concerted direct insertion, but proceeds through a linear transition state in which the original C—H distance is little increased. Subsequent rearrangement of atoms can lead directly to products (Scheme 14). Similar calculations for triplet oxene[114] indicate a conventional radical abstraction mechanism. The formation of an oxene intermediate could therefore be consistent with either pathway of Scheme 11; no information on the spin state of such an intermediate in the enzymic reaction currently exists.



Scheme 14 *Theoretical path for* C—H *hydroxylation by singlet oxene*[113,114]

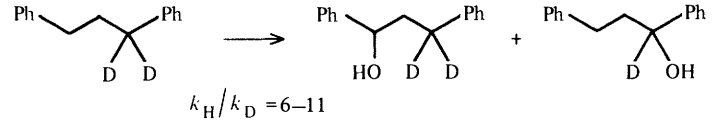
The observation of epimerization or rearrangement in several reactions militates in favour of a radical intermediate which is relatively long lived or loosely bound to the enzyme. However, the failure to observe epimerization at carbon during enzymic hydroxylation may be attributable either to a concerted process (singlet oxene) or to a triplet oxene or radical pathway in which collapse of the intermediate radical pair occurs before significant loss of configuration. Since the majority of hydroxylations occur with retention of configuration, and this situation is clearly not amenable to unambiguous mechanistic interpretation, other experimental parameters are required.

(ii) *Kinetic Isotope Effect Studies.* The rate determining step in the cytochrome P-450 cycle (Scheme 2) has been variously reported as the addition of the second electron,[115] and the decomposition of the P-450–substrate–oxygen complex to products.[116] It is not clear to what extent the later steps of the cycle of Scheme 2 are kinetically distinct, and this has hampered a clear interpretation of the intermolecular isotope effects observed for hydroxylation. In general, such effects have been determined by product composition analysis following competitive hydroxylation of both labelled and unlabelled substrates. The need to correct for the extent of reaction,[117] and the possible existence of isotope effects in substrate binding and other non rate-limiting steps[118] present additional complications.

[115] P. Shannon and T. C. Bruice, *J. Am. Chem. Soc.*, 1981, **103**, 4580.
[116] Y. Ishimura in 'Cytochrome P-450', ed. R. Sato and R. Omura, Academic Press, New York, 1978, p. 211.
[117] C. J. Collins and M. H. Lietzke, *J. Am. Chem. Soc.*, 1959, **81**, 5379.
[118] D. B. Northrop, *Ann. Rev. Biochem.*, 1981, **50**, 103.

Intermolecular isotope effects obtained in this way for steroid hydroxylations at C-6β,[71,119] C-7α,[119,120] C-7β,[82] and C-11α,[93] and also for non-steroid substrates,[121] are low (generally $k_H/k_D < 2$) and have been interpreted as consistent with a concerted insertion mechanism.[67] The analogous effects for carbene[122] and carbenoid[123] insertions are indeed low ($k_H/k_D \leq 2$) but radical abstractions may also involve isotope effects of a similar magnitude.[124,125]

In cases where both inter- and intra-molecular isotope effects have been determined for the same substrate, appreciable differences are apparent.[126] The intramolecular effects are typically large ($k_H/k_D > 5$),[84,102] exemplified in Scheme 15.[127] The only intramolecular effect so far reported for a steroid



Scheme 15 *Hydroxylation of* 1,3-*diphenyl*-[1,1-$^2H_2$]*propane*[127]

hydroxylation (k_H/k_D for C-21 hydroxylation = 1.2)[67] differs substantially from the values for non-steroid substrates. In view of the complicating factors discussed above, the intramolecular isotope effects are now viewed as potentially more useful than intermolecular effects in providing data for mechanistic interpretation.[102,118] The large effects observed are consistent with a radical abstraction process with a transition state in which the hydrogen is approximately equally shared between the atom which it is leaving and the abstracting species,[128] but are difficult to reconcile with a concerted direct insertion mechanism (*vide supra*).[86]

4 Summary

Considerable progress has been made in understanding the steroid hydroxylation reaction. The nature of the enzymically activated oxidizing species is now clear, and factors which can control the binding and possible activation of the substrate have been identified. However, the exact mechanism of the hydroxylation step is not yet fully understood. Product and isotope effect studies lend weight to a radical abstraction–recombination mechanism for the hydroxylation of non-steroid substrates, and, by extension, of steroids. The latter, however, is by no means certain and further work in this area is clearly required.

[119] I. Björkhem, *Eur. J. Biochem.*, 1971, **18**, 299.
[120] M. J. Frey, H. Jirku, and M. Levitz, *J. Labelled Comp. Radiopharm.*, 1970, **6**, 355.
[121] R. E. McMahon and H. W. Culp, *J. Med. Chem.*, 1979, **22**, 1100.
[122] V. Franzen and R. Edens, *Justus Liebigs Ann. Chem.*, 1969, **729**, 33.
[123] M. J. Goldstein and W. R. Dolbier, *J. Am. Chem. Soc.*, 1965, **87**, 2293.
[124] J. T. Groves and M. Van Der Puy, *J. Am. Chem. Soc.*, 1976, **98**, 5290.
[125] L. B. Harding and W. A. Goddard, *J. Am. Chem. Soc.*, 1980, **102**, 439.
[126] A. B. Foster, M. Jarman, J. D. Stevens, P. Thomas, and J. H. Westwood, *Chem.-Biol. Interactions*, 1974, **9**, 327.
[127] L. M. Hjelmeland, L. Aronow, and J. R. Trudell, *Biochem. Biophys. Res. Commun.*, 1977, **76**, 541.
[128] H. Simon and D. Palm, *Angew. Chem., Int. Ed. Engl.*, 1966, **5**, 920.