Methyl Group Removal in Steroid Biosynthesis

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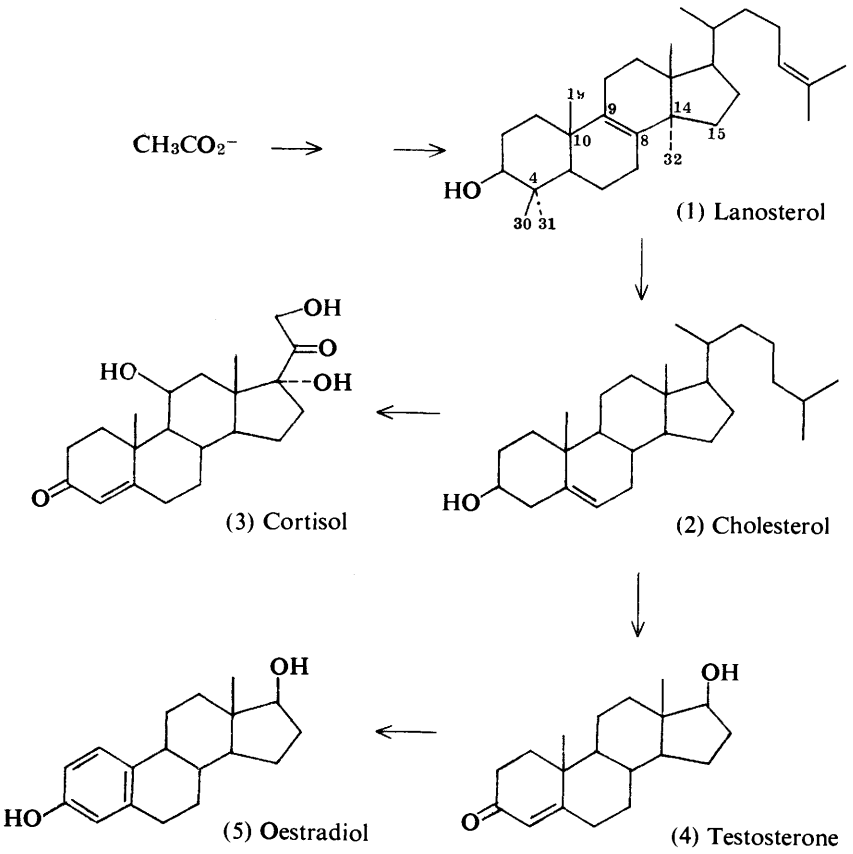
1 Introduction

A. Steroid Biosynthesis.—The biosynthesis of the physiologically active steroid hormones in mammals can be conveniently divided into two processes, as summarized in Scheme 1. Firstly, an anabolic (synthetic) conversion of acetate *via* mevalonic acid and squalene into the C_{30} molecule lanosterol (1), followed by a catabolic (degradative) conversion of lanosterol *via* cholesterol [C_{27}, (2)] into the steroid hormones such as cortisol [C_{21}, (3)], testosterone [C_{19}, (4)], and oestradiol [C_{18}, (5)].

The route by which acetate is converted into lanosterol was extensively investigated and described some time ago,[1,2] and many of the mechanistic features of this conversion have now been elucidated.[3] The transformation of lanosterol into the C_{27}, C_{21}, C_{19}, and C_{18} steroids involves a complex series of enzymic reactions, many of which are imperfectly understood at the present time. The changes brought about are: (i) the saturation of C=C bonds and introduction of new sites of unsaturation; (ii) progressive cleavage of the C-17 side-chain; (iii) the removal of the methyl groups at C-4 (C-30 and C-31) and C-14 (C-32), and for the C_{18} steroids at C-10 (C-19); (iv) the introduction of hydroxy-groups at specific sites in the steroid nucleus; and (v) keto–alcohol interconversions. The biosynthetic interconversions which are possible among the C_{21}, C_{19}, and C_{18} steroids are manifold,[4] and the above list is not intended to imply a specific sequence of events. However, it is known that the gross skeletal changes occur in the following order: lanosterol → cholesterol → pregnanes (C_{21}) → androstanes (C_{19}) → oestranes (C_{18}). These changes are brought about by both side-chain cleavage (C_{27} → C_{21} → C_{19}) and by loss of methyl groups (C_{30} → C_{27} and C_{19} → C_{18}). This review is concerned with the removal of the C-30—32 and C-19 methyl carbons, and will therefore be dealing with reactions that occur at both early (loss of C-30—C-32) and late (loss of C-19) stages of the overall pathway. The actual sequence of loss of C-30—C-32 is considered in more detail in Section 4 (below).

[1] R. B. Clayton, *Q. Rev. Chem. Soc.*, 1965, **19**, 168, 201.
[2] I. D. Franz and G. J Schroepfer, *Annu. Rev. Biochem.*, 1967, **36**, 656. T. W. Goodwin, 'Rodd's Chemistry of Carbon Compounds', 2nd Edn., ed. M. F. Ansell, Elsevier, Amsterdam, vol. 2, 1974, p. 237.
[3] D. E. Cane, *Tetrahedron*, 1980, **36**, 1109.
[4] R. I. Dorfman and F. Ungar, 'Metabolism of Steroid Hormones,' Academic Press, New York, 1965.



Scheme 1 *Biosynthesis of steroid hormones*

B. Mono-oxygenase Enzymes.—The cleavage of the C-17 side-chain and the removal of methyl groups during steroid biosynthesis are oxidative processes, catalysed by mono-oxygenases.[5] This group of enzymes catalyses the introduction of oxygen into an organic substrate according to Equation 1.

$$R—H + NADPH + O_2 \rightarrow R—OH + NADP^+ + OH^- \quad (1)$$

The key features of this conversion are: (i) the introduction of one atom of molecular oxygen into R—H, the other being incorporated into a molecule of water; (ii) the requirement of one equivalent of an NADPH co-factor; (iii) the apparent absence of any required activation of the C—H bond of the substrate; and (iv) the involvement (not shown in Equation 1) of a cytochrome co-factor.

[5] M. J. Coon and R. E. White, in 'Metal Ion Activation of Dioxygen,' ed. T. G. Spiro, Wiley, New York, 1980, p. 73.

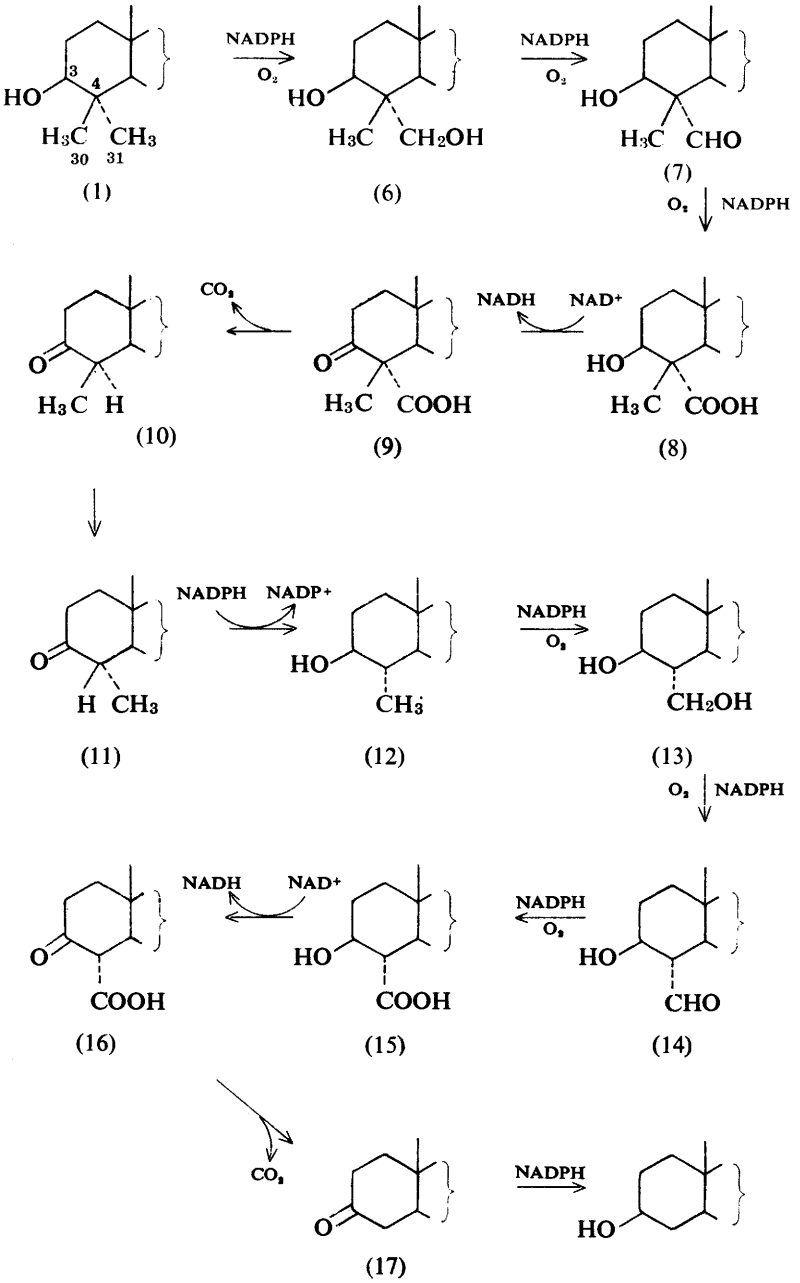
2 Removal of Methyl Groups from C-4 of Lanosterol

Of the methyl-group removals in steroid biosynthesis, those from C-4 are perhaps the best understood. Pioneering investigation by Bloch and co-workers[6] in the 1950's established that rat liver homogenates were able efficiently to convert lanosterol (1) into cholesterol (2), that the reaction required molecular oxygen and NADPH, and that the methyl carbons were released as carbon dioxide (but *vide infra*, Section 3). It stands as a tribute to the insight of this group that the pathway for the lanosterol–cholesterol conversion proposed by them in 1956 contained many of the features of the currently accepted route for C-4 demethylation (Scheme 2). The latter was elucidated almost a decade ago, largely by the efforts of Gaylor, Sharpless, and Clayton and their co-workers.[7–11]

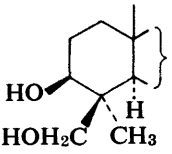
The route shown in Scheme 2 was deduced by the classical methods of isolation of intermediates, and incubation of these and other postulated intermediates with an enzymic system capable of performing the overall transformation. The isolated intermediates include 3β-hydroxy-4β-methyl-4α-carboxylic acids (8),[11–13] 4-α-methyl-3-keto-steroids (11),[14,15] a 3β-hydroxy-4α-methyl-steroid (12),[9] and a 3β-hydroxy-4α-carboxylic acid (15).[16] Efficient transformation into 4-demethyl-steroids has been observed with a 3β-hydroxy-4β-methyl-4α-carboxylic acid (8),[15] a 4α-methyl-3-keto-steroid (11),[17] 3β-hydroxy-4α-methyl-steroids (12),[11,14,16,17] a 3β-hydroxy-4α-hydroxymethyl-steroid (13),[8] a 3β-hydroxy-4α-formyl-steroid (14),[18] and a 3β-hydroxy-4α-carboxylic acid (15).[15]

Crucial evidence for the sequence of events in this pathway was provided first by Sharpless and co-workers,[8] who demonstrated that a 4β-methyl-4α-hydroxy-methyl-steroid (6) was converted efficiently into cholestanol by a rat liver homogenate, whereas the isomeric 4α-methyl-4β-hydroxymethyl-steroid (18) was not metabolized. This firmly established the initial site of oxidation as the C-4α methyl group (C-31). The next identified intermediate in the pathway, the carboxylic acid (8), was obtained from incubations in which the enzyme co-factor NAD^+ was absent.[12] The significance of the 3-keto-intermediates (9) and (16) was deduced from a requirement of the overall conversion for the alcohol dehydrogenase co-factor NAD^+,[11] necessary for oxidation of the C-3β alcohol group.[16,19]

[6] J. A. Olsen, M. Lindberg, and K. Bloch, *J. Biol. Chem.*, 1957, **226**, 941.
[7] J. L. Gaylor and C. V. Delwiche, *Steroids*, 1964, **4**, 207.
[8] K. B. Sharpless, T. E. Synder, T. A. Spencer, K. K. Maheshwari, G. Guhn, and R. B. Clayton, *J. Am. Chem. Soc.*, 1968, **90**, 6874.
[9] R. Rahman, K. B. Sharpless, T. A. Spencer, and R. B. Clayton, *J. Biol. Chem.*, 1970, **245**, 2667.
[10] E. L. Ghisalberti, N. J. DeSouza, H. H. Rees, L. J. Goad, and T. W. Goodwin, *Chem. Comm.*, 1969, 1403.
[11] W. L. Miller and J. L. Gaylor, *J. Biol. Chem.*, 1970, **245**, 5375.
[12] G. M. Hornby and G. S Boyd, *Biochem. Biophys. Res. Commun.*, 1970, **40**, 1452.
[13] D. P. Bloxham and M. Akhtar, *Biochem. J.*, 1971, **123**, 275.
[14] A. C. Swindell and J. L. Gaylor, *J. Biol. Chem.*, 1968, **243**, 5546.
[15] A. D. Rahimtula and J. L. Gaylor, *J. Biol. Chem.*, 1972, **247**, 9.
[16] W. L. Miller and J. L. Gaylor, *J. Biol. Chem.*, 1970, **245**, 5369.
[17] K. B. Sharpless, T. E. Synder, T. A. Spencer, K. K. Maheshwari, J. A. Nelson, and R. B. Clayton, *J. Am. Chem. Soc.*, 1969, **91**, 3394.
[18] D. R. Brady, R. D. Crowder, and W. Ja Hayes, *J. Biol. Chem.*, 1980, **255**, 10624.
[19] N. J. Moir, W. L. Miller, and J. L. Gaylor, *Biochem. Biophys. Res. Commun.*, 1968, **33**, 916.



Scheme 2 *Removal of the methyl groups from* C-4 *of lanosterol*



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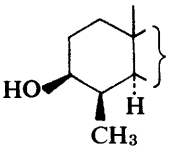


(19)

The presence of a carbonyl group at C-3 serves not only to facilitate the loss of carbon dioxide from the resulting β-keto-acids (9) and (16),[20] but also to effect, *via* enolization, the epimerization of the C-4β methyl group of (10) to the α-position.[15] It has been demonstrated that oxidative attack at the C-4 methyl groups occurs only from the α-side: a C-4β monomethyl-steroid such as (19) is not transformed by the enzymes of this pathway.[17]

A requirement for exogenous NADPH, and the observation[14] that, in its absence, C-3 keto-steroids such as (11) are not transformed, led to the conclusion that the β-alcohol functionality at C-3 must be regenerated by an NADPH dependent reductase before oxidation of C-4 monomethyl-steroids can proceed. The absence of NAD^+ leads to the accumulation of the carboxylic acid (15), in addition to (8),[15] implicating a further oxidation of the C-3 alcohol to carbonyl before decarboxylation occurs to produce (17). The final step in the sequence, the reduction of the C-3 carbonyl of (17) to alcohol, also requires NADPH.[15]

Several of the enzymes which catalyse reactions of Scheme 2 have been partially purified and characterized by Gaylor and co-workers, and data thus obtained have been of decisive value in the elucidation of the sequence of reactions portrayed in that Scheme. In spite of a requirement for NADPH and molecular oxygen, the oxidative enzymes in rat liver capable of demethylating 4,4-dimethyl-cholest-7-ene-3β-ol at C-4 do not require cytochrome P-450 or cytochrome b_5,[21] both of which are common mono-oxygenase co-factors:[5] the identities of the electron transfer components of the C-4 methyl-sterol oxidases are unknown at the present time. The alcohol dehydrogenase which catalyses oxidation at C-3 during demethylation has been partially purified and characterized.[15] Indirect evidence suggests that this oxidation is rate limiting in the decarboxylation of (8) → (10), and that the latter may in fact be a non-enzymic process at the pH (9.0) optimal for the activity of the dehydrogenase.[15,20]

3 Removal of the C-14α Methyl Group

In spite of having been subjected to the same intense effort as that expended upon demethylation at C-4, the C-14α demethylation reaction remains incompletely understood. It was originally proposed[6] that the methyl carbon was liberated as carbon dioxide, but later work[22] demonstrated that, in contrast to the losses from

[20] M. Lindberg, F. Gautschi, and K. Bloch, *J. Biol. Chem.*, 1963, **238**, 1661.
[21] J. L. Gaylor and H. S. Mason, *J. Biol. Chem.*, 1968, **243**, 4966.
[22] K. Alexander, M. Akhtar, R. B. Boar, J. F. McGhie, and D. H. R. Barton, *J. Chem. Soc., Chem. Commun.*, 1972, 383.

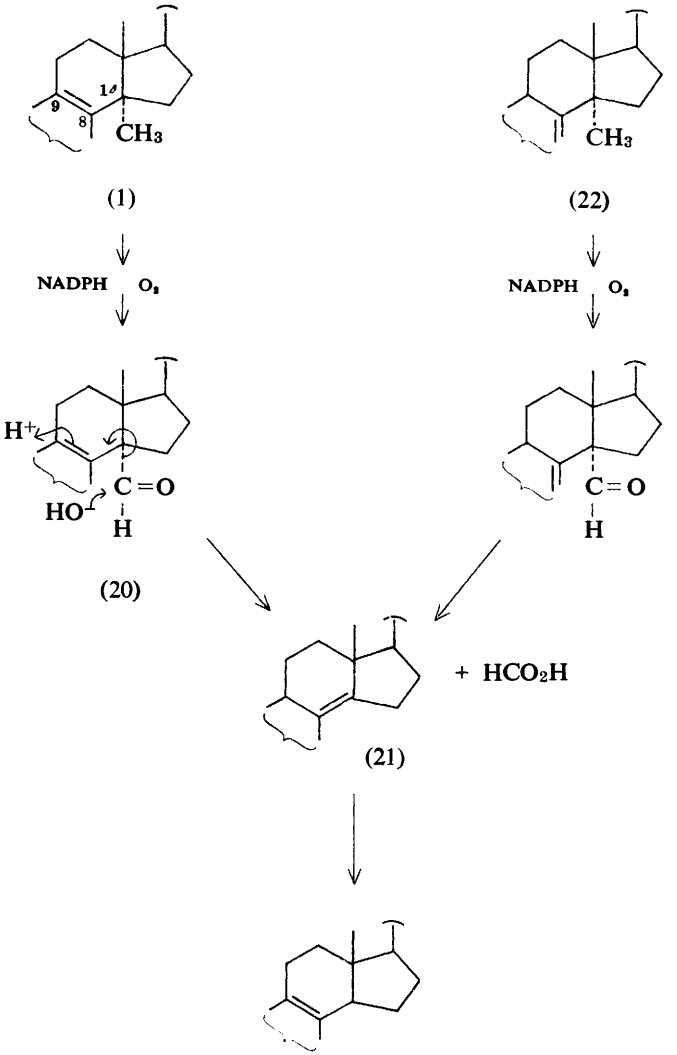
C-4, the loss of C-32 from the C-14α position occurs at the aldehyde oxidation level, resulting in the release of formic acid. This was subsequently oxidized to carbon dioxide by other enzymes present in the liver homogenates used.[23] Also in contrast to C-4 demethylation, the removal of the C-14α methyl requires a cytochrome P-450 co-factor[24] in addition to NADPH and molecular oxygen, although it appears that the cytochrome P-450 may be necessary only for the initial oxidative step (—CH_3 → —CH_2OH).[25]

It was suggested some time ago that loss of methyl from C-14 was accompanied by the loss of hydrogen from C-15β,[26] but it was later demonstrated unequivocally that the loss occurs from the C-15α position,[27] the original assignments of stereochemistry of label having been in error. No loss of hydrogen from the C-16 position occurs during the conversion of lanosterol into cholesterol by rat liver homogenate.[28]

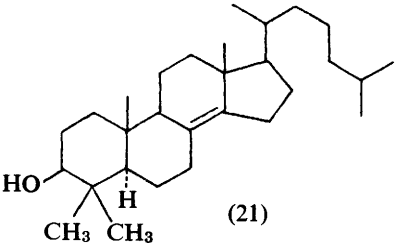
The possible role of a $\Delta^{8(9)}$ or Δ^{7} double bond in C-14α demethylation has received much attention. The presence of unsaturation in a position β,γ to the carbon that is lost led to the postulate[29] that the activation so achieved may be instrumental in promoting the loss of C-32 as formic acid, as shown in Scheme 3. However, the observations[30] that the $\Delta^{8(14)}$ steroid (21) is only poorly converted into cholesterol, and that conversion of the C-14α aldehyde (20) into cholesterol requires NADPH and oxygen, together with the observed loss of the C-15α hydrogen discussed above, have been cited[30] as evidence against this proposal.

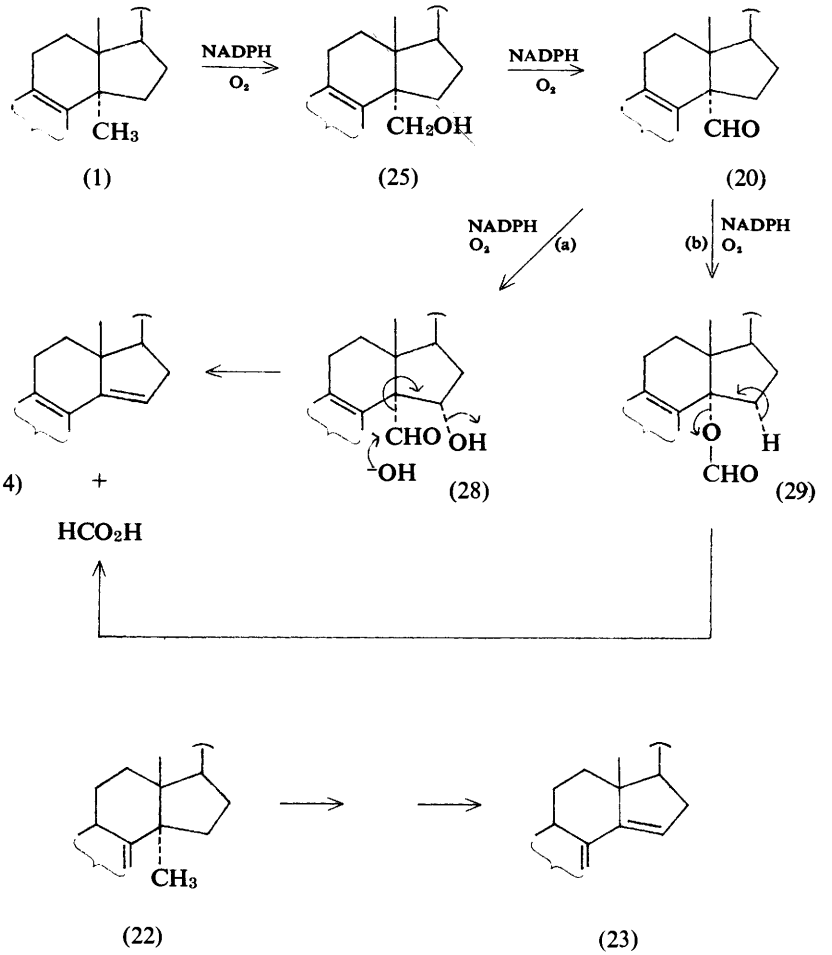
An observation of significance with respect to the loss of hydrogen from C-15 is that the initial products of enzymic C-14α demethylation of C_{30} steroids with a Δ^{7} (22) or $\Delta^{8(9)}$ (1) double bonds are the corresponding $\Delta^{7,14}$- and $\Delta^{8(9),14}$-dienes, (23) and (24), respectively.[30] This led to the proposal of the mechanisms shown in Scheme 4,[30] for which the following evidence was cited. Firstly, C-14α hydroxymethyl intermediates of type (25) are dealkylated efficiently by rat liver preparations, liberating C-32 as formic acid[30–32] and the corresponding aldehyde (20) is similarly transformed.[30,32] The liberation of formic acid from (25) and the production of C-14α demethyl-steroids was reported to be dependent upon the presence of NADPH and oxygen, no significant transformation occurring when this combination was replaced by $NADP^+$, NAD^+, or NADPH under nitrogen.[30]

[23] S. Trowbridge, Y. C. Lu, R. Shaw, J. Chan, and T. Spike, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 1975, **34**, 560.
[24] G. F. Gibbons and K. A. Mitropoulos, *Eur. J. Biochem.*, 1973, **40**, 267.
[25] G. F. Gibbons, C. R. Pullinger, and K. A. Mitropoulos, *Biochem. J.*, 1979, **183**, 309.
[26] L. Canonica, A. Fiecchi, M. Galli Kienle, A. Scala, G. Galli, E. Grossi Paoletti, and R. Paoletti, *J. Am. Chem. Soc.*, 1968, **90**, 3597.
[27] G. F. Gibbons, L. J. Goad, and T. W. Goodwin, *Chem. Commun.*, 1968, 1458.
[28] M. Akhtar, A. D. Rahimtula, I. A. Watkinson, D. C. Wilton, and K. A. Munday, *Eur. J. Biochem.*, 1969, **9**, 107.
[29] L. Richards and J. B. Hendrickson, 'The Biosynthesis of Sterols, Terpenes, and Acetogenins,' Benjamin, New York, 1964.
[30] M. Akhtar, K. Alexander, R. B. Boar, J. F. McGhie, and D. H. R. Barton, *Biochem. J.*, 1978, **169**, 449.
[31] M. Akhtar, C. W. Freeman, D. C. Wilton, R. B. Boar, and D. B. Copsey, *Bioorg. Chem.*, 1977, **6**, 473.
[32] J. Fried, A. Dudowitz, and J. W. Brown, *Biochem. Biophys. Res. Commun.*, 1968, **32**, 568.



Scheme 3 *Loss of* C-32 *from lanosterol involving a* $\Delta^{8(9)}$ *or* Δ^{7} *bond*





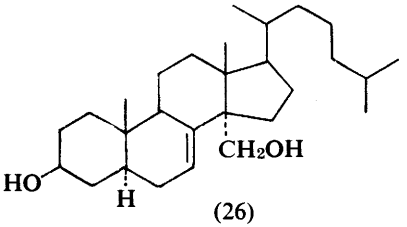
Scheme 4 *Formation of dienes on removal of* C-32 *from* C_{30} *sterols**

*Illustrated in detail only for a $\Delta^{8(9)}$ substrate. A similar mechanistic proposal applies to Δ^{7} substrates, yielding $\Delta^{7,14}$ dienes.

Akhtar and Barton and their co-workers[30] also established that the conversion of (25) into (20) required NADPH (and, by extension, oxygen). This conversion has been stated to occur in the presence of only NAD^+ under nitrogen or helium,[33] but the absence of endogenous NADPH and absorbed oxygen, which could support the reaction, was not established in this case. The work of Akhtar *et al.*[30] which demonstrates the accumulation of the aldehyde (20) under conditions of limiting NADPH concentration suggests that NADPH (and, by inference, oxygen) may be required for further conversion of (20). The liberation of

[33] A. E. Dudowitz and J. Fried, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 1969, **28**, 665.

formic acid from the aldehyde (20) also appears to be dependent upon NADPH and oxygen.[30,33] However, a recent report[34] states that the 4,4-demethyl-14α-hydroxymethyl-Δ^7-steroid (26) is converted into the $\Delta^{8(14)}$-ene (27) by liver



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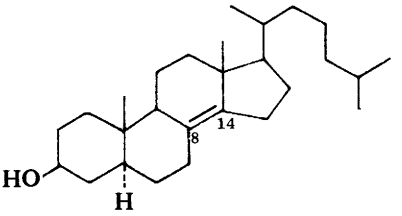


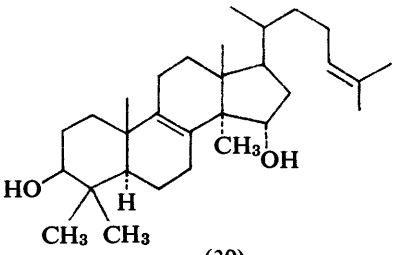
(27)

microsomes under anaerobic conditions and with only NAD^+ as a co-factor. On the basis of this and other evidence, the latter workers[34] have indicated support for the mechanism of C-32 removal outlined in Scheme 3, but offer no explanation for the observed loss of hydrogen from C-15α of lanosterol. This loss, however, has not been demonstrated for 4,4-demethyl C_{28} substrates. In addition, the product obtained in this study contained only an isolated $\Delta^{8(14)}$ double bond; the formation of conjugated dienes such as (23) and (24), obtained by Akhtar *et al.*,[30] was not observed. These results, obtained with similar enzyme preparations, are obviously at variance; the differences may be attributable to the use of C_{28} substrates, devoid of the C-4 methyl groups, in the more recent study,[34] but the reasons for them are not clear. Both 4,4-demethyl-$\Delta^{8(14)}$-enes [e.g. (27)][35] and $\Delta^{8(9),14}$-dienes [e.g. (24)][36] are further metabolized to cholesterol by rat liver preparations. However, since C_{27} mono-enes [e.g. (27)] have only been obtained as a product when the substrate [e.g. (26)] is devoid of methyl groups at C-4, and are only efficiently converted into cholesterol in the absence of these substituents,[30,34] and the co-factor requirements for these conversions may be different, the influence of the C-4 methyl groups on the process of C-14α demethylation may be significant. The ultimate interpretation of the available mechanistic data on the role and nature of unsaturation at carbons 7, 8, 9, 14, and 15 during C-14α demethylation may therefore depend upon the rigid establishment of the sequence of demethylation of C-4 and C-14α of lanosterol (see Section 4).

The later stages of Scheme 4 [path (a)] included an intermediate oxygenated at C-15 (28). The possibility that C-15 oxygenated steroids may be intermediates in the C-14α demethylation reaction was suggested by the loss of the C-15α hydrogen from lanosterol during its conversion into cholesterol. The apparent requirement for NADPH and oxygen of the dealkylation of (20)[30,33] is consistent with

[34] R. A. Pascal, P. Chang, and G. J. Schroepfer, *J. Am. Chem. Soc.*, 1980, **102**, 6599.
[35] G. J. Schroepfer, B. N. Lutsky, J. A. Martin, S. Huntoon, B. Fourcans, W.-H. Lee, and J. Vermilion, *Proc. R. Soc. London, Ser. B*, 1972, **180**, 125.
[36] A. Fiecchi, L. Canonica, A. Scala, F. Cattabeni, E. Grossi Paoletti, and R. Paoletti, *Life Sciences*, 1969, **8**, 629.

the involvement in this step of a mono-oxygenase enzyme; enzymes of this type are known to hydroxylate steroids with retention of stereochemistry.[37] However, although the 15α-hydroxy-4,4-dimethyl-steroid (30) gives rise to C_{27} steroids on incubation with rat liver homogenate, so does its C-15β epimer (31), and the



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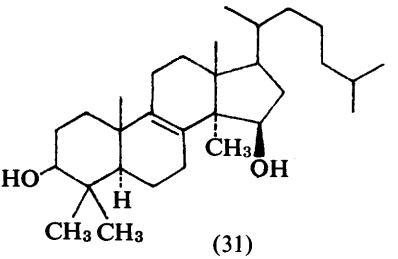

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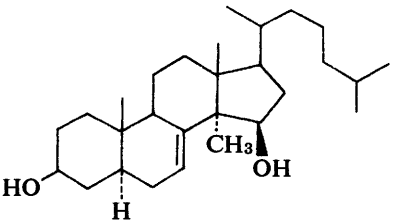
conversion efficiencies are low, both being extensively metabolized by other routes.[38] The conversion of 14α-methyl-Δ^7-cholestene-3β,15β-diol (32) into



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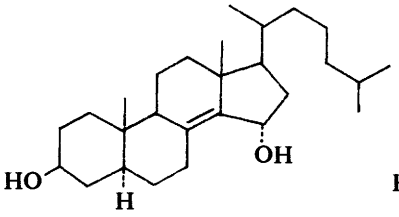
cholesterol by a rat liver homogenate preparation has been reported,[39,40] but the intermediacy of a C-15β alcohol is inconsistent with the observed loss (from C_{30} substrates) of the C-15α hydrogen occurring by a mono-oxygenase catalysed oxidation with retention of configuration. The possibility that this hydroxylation may proceed with inversion of configuration has been suggested,[40] but there is lack of adequate precedent for this. Both the 15α- and 15β-hydroxycholestenes (33) and (34),[41] and the corresponding ketone (35)[42] are converted efficiently into C-15 deoxygenated C_{27} steroids, including cholesterol (2), by rat liver homo-

[37] L. L. Smith in 'Terpenoids and Steroids,' A Specialist Periodical Report, The Chemical Society, London, vol. 4, 1974, p. 394.
[38] G. F. Gibbons, K. A. Mitropoulous, and C. R. Pullinger, *Biochem. Biophys. Res. Commun.*, 1976, **69**, 781.
[39] J. A. Martin, S. Huntoon, and G. J. Schroepfer, *Biochem. Biophys. Res. Commun.*, 1970, **39**, 1170.
[40] T. E. Spike, A. H.-J. Wang, I. C. Paul, and G. J. Schroepfer, *J. Chem. Soc., Chem. Commun.*, 1974, 477.
[41] S. Huntoon, B. Fourcans, B. N. Lutsky, E. J. Parish, H. Emery, F. F. Knapp, and G. J. Schroepfer, *J. Biol. Chem.*, 1978, **253**, 775.
[42] D. J. Monger, E. J. Parish, and G. J. Schroepfer, *J. Biol. Chem.*, 1980, **255**, 11122.



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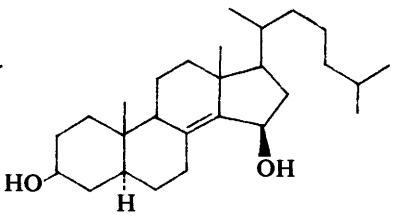

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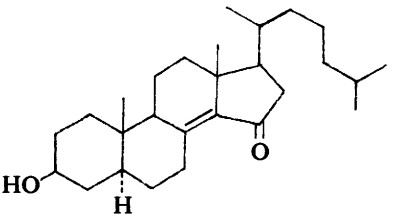


(35)

genates, but the relevance of these findings to the possible involvement of C-15 oxygenated intermediates in C-14α demethylation is not clear.

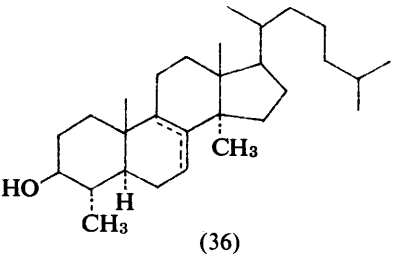
An alternative pathway to account for the loss of the C-15α hydrogen, path (b) (Scheme 4), involves stereospecific loss of this hydrogen from the C-14α formate (29), produced by an enzymic 'Baeyer–Villiger' reaction on the aldehyde (20). Similar oxidative reactions are thought[37] to be involved in the cleavage of the steroid side-chain between C-20 and C-17, and may also be involved in the loss of the C-19 carbon in oestrogen biosynthesis (see Section 5). In the absence of any definitive data on the proposed intermediates (28) and (29), however, their involvement in the C-14α demethylation of lanosterol is still speculative.

4 Sequence of the Demethylations at C-4 and C-14α

As discussed in the preceding section, the presence or absence of methyl groups at C-4 in substrates which have been employed in the study of C-14α demethylation can lead to differing conclusions regarding the mechanism of the latter transformation. It is therefore of some concern that the sequence of demethylation of C_{30} precursors be firmly established. Many of the substrates used in the study of C-4 demethylation (Section 2) were 4-methyl- or 4,4-dimethyl-cholesterol derivatives, devoid of the C-14α methyl. This has been justified by the common assumption that demethylation at C-14α preceeds that at C-4, an assumption based on the isolation of C-14α monodemethyl-steroids from both mammalian[43–45] and micro-organism[45] sources capable of synthesizing cholesterol and ergosterol, respectively. However, one of these reports[44] also established the

[43] F. Gautschi and K. Bloch, *J. Am. Chem. Soc.*, 1957, **79**, 684.
[44] J.-A. Gustafsson and P. Eneroth, *Proc. R. Soc. London, Ser. B*, 1972, **180**, 179.
[45] K. A. Mitropoulos, G. F. Gibbons, and B. E. A. Reeves, *Steroids*, 1976, **27**, 821.

presence in meconium from newborn infants of the 4β-monodemethyl-steroid (36) [*cf.* part structure (12), Scheme 2], a result which has been quoted[34] in support

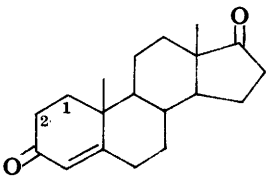


(36)

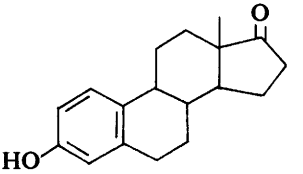
of a pathway for cholesterol biosynthesis in which demethylation commences at C-4, and subsequent loss of the C-14 methyl group occurs as outlined in Scheme 3. Other reports of the isolation of 4-demethyl-14α-methyl-steroids concern plant[46–48] or yeast mutant[49] sources, or enzyme systems operating in the presence of specific inhibitors;[50–52] the relevance of these findings to the normal pathway for cholesterol biosynthesis in mammals remains to be established. The available evidence therefore favours C-14α as the first site of demethylation of lanosterol, but in the absence of definitive data available from purified enzyme systems capable of performing this reaction, this conclusion must be regarded as tentative.

5 Removal of C-19 and the Mechanism of Oestrogen Biosynthesis

The conversion of C_{19} steroids such as testosterone (4) or 4-androstene-3,17-dione (37) into the corresponding C_{18} hormones oestradiol (5) and oestrone (38)



(37)



(38)

involves both removal of the C-19 methyl group from C-10 and the aromatization of ring A. Both (4) and (37) have been used as substrates in the study of this transformation, which is normally carried out using a microsomal preparation

[46] C. Djerassi, J. C. Knight, and D. I. Wilkinson, *J. Am. Chem. Soc.*, 1963, **85**, 835.
[47] L. J. Goad, B. L. Williams, and T. W. Goodwin, *Eur. J. Biochem.*, 1967, **3**, 232.
[48] A. M. Atallah and H. J. Nicholas, *Steroids*, 1981, **17**, 611.
[49] P. J. Trocha, S. J. Jasne, and D. B. Sprinson, *Biochemistry*, 1977, **16**, 4721.
[50] P. J. Doyle, G. W. Patterson, S. R. Dutky, and C. F. Cohen, *Phytochemistry*, 1971, **10**, 2093.
[51] G. F. Gibbons and K. A. Mitropoulous, *Biochem. J.*, 1973, **132**, 439.
[52] N. N. Ragsdale, *Biochim. Biophys. Acta*, 1975, **380**, 81.

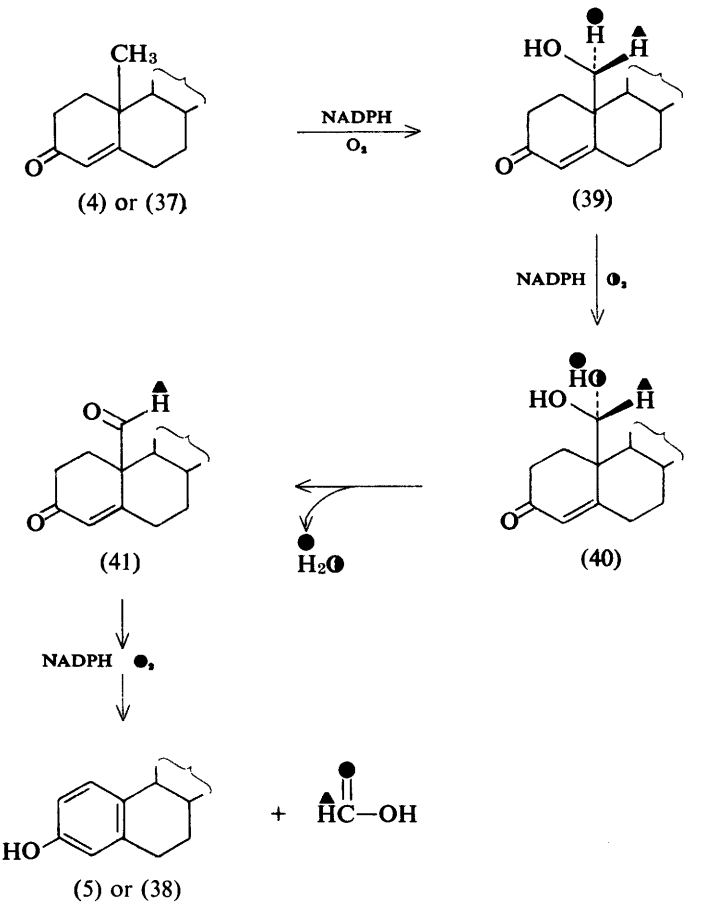
from human placenta; the available evidence indicates that both are transformed at the same enzyme site by the same mechanism,[53,54] and may be interconverted during the transformation by reversible oxidation–reduction at C-17.[55]

Although the initial stages of methyl group removal have been thoroughly investigated, later reactions of the aromatization sequence are not so well understood. The overall transformation of (4) or (37) to the corresponding oestrogenic steroid requires three equivalents of NADPH and molecular oxygen,[56] indicating the involvement of a mono-oxygenase enzyme. As is the case for C-4 demethylation, a requirement for a cytochrome P-450 co-factor has not been rigidly established for the aromatization reaction. The latter conversion is insensitive to carbon monoxide, an effective inhibitor of cytochrome P-450-dependent oxidations, suggesting the involvement of either a different electron carrier or a CO-insensitive form of cytochrome P-450.[57]

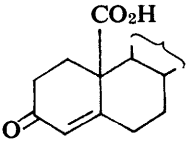
Two of the required oxidizing equivalents are consumed in the conversion of C-19 from the methyl into the aldehyde oxidation levels, as outlined in Scheme 5. The intermediacy of the C-19 alcohol (39) and the aldehyde (41), the latter formed by collapse of the *gem*-diol (40), has been confirmed by their isolation,[58,59] their ability to act as substrates for the aromatase enzyme system,[55,60,61] and their effectiveness as competitive inhibitors of the normal substrate (4) or (37).[61] The loss of C-19 has been demonstrated to occur as formic acid;[62] the C-19 carboxylic acid (42) is not aromatized by placental microsomes.[63] The stereochemical fate of the hydrogens involved was determined to be that shown in Scheme 5,[64,65] and the fate of the oxygen atoms involved in the oxidation at C-19 was deduced by Akhtar and co-workers from experiments with ^{18}O-labelled materials.[66,67]

The outstanding problems in oestrogen biosynthesis concern the function of the third oxidizing equivalent and the pathway by which the C-19 aldehyde (41) is aromatized. Available evidence from the use of labelled substrates has con-

[53] W. Gibb and J.-C. Lavoie, *Steroids*, 1980, **36**, 507.
[54] K. C. Reed and S. Ohno, *J. Biol. Chem.*, 1976, **251**, 1625.
[55] W. E. Braselton, L. L. Engel, and J. C. Orr, *Eur. J. Biochem.*, 1974, **48**, 35.
[56] E. A. Thompson and P. K. Siiteri, *J. Biol. Chem.*, 1974, **249**, 5364.
[57] R. A. Meigs and K. J. Ryan, *Biochim. Biophys. Acta*, 1968, **165**, 476.
[58] A. S. Meyer, *Experientia*, 1955, **11**, 99.
[59] M. Akhtar and S. J. M. Skinner, *Biochem. J.*, 1968, **109**, 318.
[60] A. S. Meyer, *Biochim. Biophys. Acta*, 1955, **17**, 441.
[61] N. Hollander, *Endocrinology*, 1962, **71**, 723; W. G. Kelly, D. Judd, and A. Stolee, *Biochemistry*, 1977, **16**, 140.
[62] L. R. Axelrod, C. Matthijssen, P. N. Rao, and J. W. Goldzieher, *Acta Endocrinol.*, 1965, **48**, 383.
[63] S. Dell'Acqua, S. Mancuso, N. Wiquist, J. L. Ruse, S. Soloman, and E. Diczfalusy, *Acta Endocrinol.*, 1967, **55**, 389.
[64] D. Arigoni, R. Battaglia, M. Akhtar, and T. Smith, *J. Chem. Soc., Chem. Commun.*, 1975, 185.
[65] E. Caspi, E. Santaniello, K. Patel, T. Arunachalam, and C. Eck, *J. Am. Chem. Soc.*, 1978, **100**, 5223.
[66] M. Akhtar, M. R. Calder, D. L. Corina, and J. N. Wright, *J. Chem. Soc., Chem. Commun.*, 1981, 129.
[67] M. Akhtar, D. Corina, J. Pratt, and T. Smith, *J. Chem. Soc., Chem. Commun.*, 1976, 854.

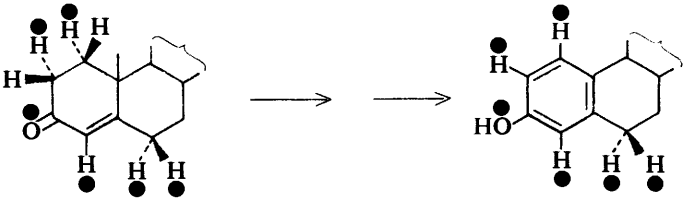


Scheme 5 *Oxidation at the* C-10 *methyl group in the aromatization of* C_{19} *steroids*



(42)

firmed the retention of oxygen at C-3,[68] eliminating the involvement of Schiff base intermediates, and the retention of hydrogens at C-1α,[69] C-2α,[70] C-4, C-6α, and C-6β[71] (Scheme 6). Pathways and intermediates that have been proposed in

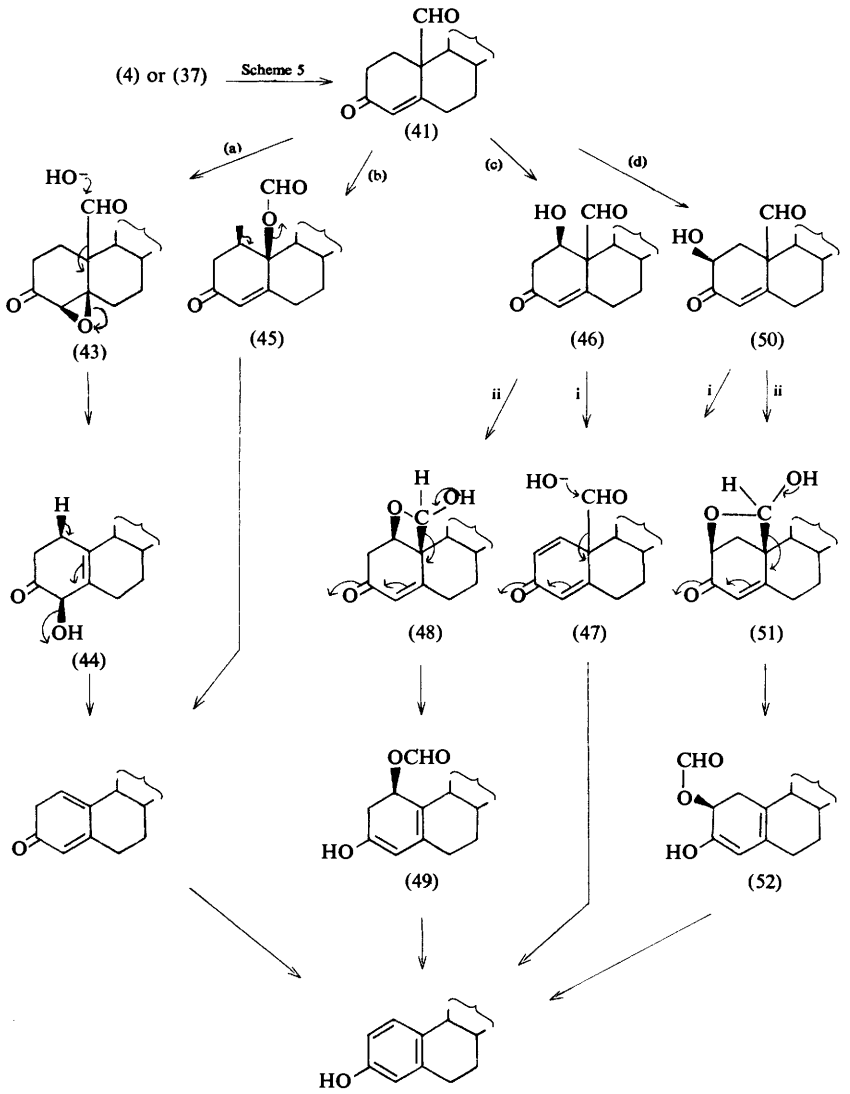


Scheme 6 *Retention of ring* A *and* B *substituents* (●) *during enzymic aromatization of* C_{19} *steroids.*

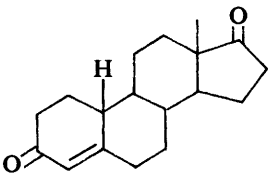
recent years for the aromatization of (41) are presented in Scheme 7. Pathway (a)[72] involves a 4,5β-epoxy-intermediate (43), of which the conversion into oestradiol has been reported.[73] However, the recent observation[66] that the oxygen atom introduced on oxidation of (41) is incorporated into the formic acid so liberated, and not into the aqueous medium (see Scheme 5), militates against the intermediacy of (43) and its derived alcohol (44), the C-4 oxygen of which must be lost to the aqueous environment.

The observed fate of the oxygen introduced during the third oxidation step would seem to support the intermediacy of the C-10β formate ester (45), formed by an enzymic Baeyer–Villiger oxidation (*cf.* Section 3). However, (45) is not aromatized by placental microsomes.[66] Nevertheless, the existence of pathway (b) and the intermediacy of a structure such as (45), perhaps as an enzyme-bound species, cannot be totally disregarded on this evidence alone (*vide infra*). An alternative pathway (c) involves a 1β-hydroxy-steroid (46). This has been proposed on the basis of the observation that human placental microsomes are capable of C-1β hydroxylation of the 19-nor-steroid (53).[74] However, the same transformation of C_{19} substrates has yet to be reported. If pathway (c) is operative, then the results of Akhtar and co-workers[66] demonstrating incorporation of the third oxidizing equivalent of oxygen into formic acid eliminate from consideration a simple dehydration of (46) *via* (47) [pathway (c, i)], and require the formation of the internal hemi-acetal (48) and the C-1β formate (49) [pathway (c, ii)]. The formation of (48) is stereochemically improbable, and in the absence

[68] H. L. Holland and G. J. Taylor, *Can. J. Chem.*, 1980, **58**, 2326.
[69] J. Fishman and M. S. Raju, *J. Biol. Chem.*, 1981, **256**, 4472.
[70] J. Fishman, H. Guzik, and D. Dixon, *Biochemistry*, 1969, **8**, 4304.
[71] H. L. Holland and G. J. Taylor, *Can. J. Chem.*, 1981, **59**, 2809.
[72] P. Morand, M. Kalapurackal, L. Lompa-Krzymien, and A. Van Tongerloo, *J. Theoret. Biol.*, 1976, **56**, 503.
[73] P. Morand, D. G. Williamson, D. S. Layne, L. Lompa-Krzymien, and J. Salvador, *Biochemistry*, 1975, **14**, 635.
[74] M. Ganguly, K. L. Cheo, and H. J. Brodie, *Biochim. Biophys. Acta*, 1976, **431**, 326.



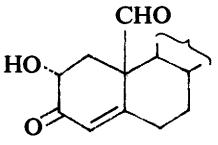
Scheme 7 *Proposed routes for the aromatization of* (41)



(53)

of further supportive data, pathway (c) must be regarded as an unattractive option at the present time.

In pathway (d), Scheme 7, aromatization occurs *via* the C-2β-hydroxy-derivative (50). In order to be consistent with the data from the oxygen labelling experiments discussed above,[66] pathway (d, i) must now be eliminated from consideration, and discussion centred on pathway (d, ii) involving the hemiacetal (51) and the 19-nor-2β-formate (52). Human placental microsomes are capable of hydroxylation of (53) at C-2β,[75] and indeed the 2β-hydroxy-intermediate (50) has been trapped (in very low yield) from incubations of labelled (37) with placental microsomes.[76] The C-2β alcohol (50) rapidly aromatizes non-enzymically at neutral or basic pH, but the C-2α alcohol (54) does not;[77]

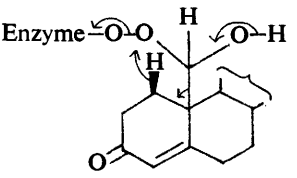


(54)

in the presence of placental microsomes, the conversion occurs with stereospecific loss of the C-1β hydrogen.[69] The alcohol (50) forms the internal hemiacetal (51) very readily;[77] this reaction has not been invoked in explaining the facile non-enzymic aromatization of (50), but may be involved in the enzymic process.

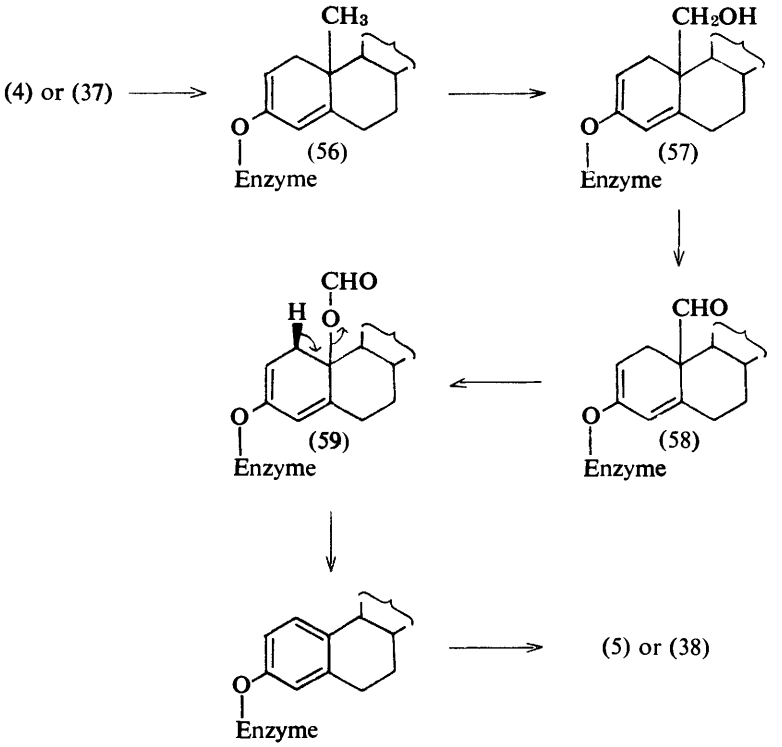
The data that are currently available therefore do not allow for a clear distinction between pathway (d, ii), and pathway (b), Scheme 7. The latter route, if operative, must involve the intermediacy of the formate (45) or its equivalent as a tightly bound intermediate, which is not in equilibium with the corresponding exogenous material (*vide supra*). Akhtar and co-workers[66] have proposed that this occurs in the form of the enzymic peroxy-acetal (55), which decomposes as shown with concerted loss of the C-1β hydrogen. An alternative route is proposed in Scheme 8. This involves binding of the substrate to the enzyme as the $\Delta^{2,4}$-dienol

[75] H. J. Brodie, A. K. Pillai, and C. E. Hay, *Biochim. Biophys. Acta*, 1969, **187**, 275.
[76] J. Goto and H. Fishman, *Science*, 1977, **195**, 80.
[77] H. Hosoda and J. Fishman, *J. Am. Chem. Soc.*, 1974, **96**, 7325.



(55)

(56), a process which would result in the observed stereospecific loss of the C-2β (axial) hydrogen.[78] The C-19 alcohol and aldehyde intermediates, (57) and (58), respectively, are known to be present as tightly enzyme-bound species,[61] not in rapid equilibium with corresponding exogenous material. A similar phenomenon for (59) may prevent it from acting as an effective substrate for aromatization when added exogenously to the enzyme system.



Scheme 8 *Proposed route for oestrogen biosynthesis* via *a* C-10β *formate ester*

[78] E. J. Corey and R. A. Sneen, *J. Am. Chem. Soc.*, 1956, **78**, 6269.

6 Summary and Comparison of Demethylation Reactions in Steroid Biosynthesis

From the data presented in Sections 2, 3, and 5 it is apparent that the loss of the C-4 methyl groups may be distinguished mechanistically from other methyl losses. The enzymic strategy of using the C-3 carbonyl group as an electron acceptor in a β-keto-acid decarboxylation necessitates the loss of carbon from C-4 as carbon dioxide. Losses from C-14 and C-10 occur as formate, in what may be formally regarded as a retro-Claisen reaction, the electron acceptors being a proton and the C-3 carbonyl of a Δ^4-3-keto-system, respectively; or these losses may occur by parallel pathways involving oxidation of the methyl group to the aldehyde level followed by Baeyer–Villiger oxidation to produce a formate ester, which then loses the elements of formic acid in a 1,2-elimination. In this event, the loss of carbon from C-10 and C-14 would not require activation by the presence of an adjacent functional group.

Although all three of the methyl group removals discussed herein require molecular oxygen and NADPH, an absolute requirement for a cytochrome P-450 electron carrier co-factor has been demonstrated only in the case of C-14α demethylation. The involvement of cytochrome P-450 in the removal of C-19 has been proposed,[79] but remains to be rigidly established, whereas its presence as a co-factor in the C-4 demethylating system has been excluded.

I wish to thank sincerely my former co-worker, G. J. Taylor, whose interest and enthusiasm in oestrogen biosynthesis was instrumental in the conception of this review.

[79] E. A. Thompson an P. K. Siiteri, *Ann. N.Y. Acad. Sci.*, 1973, **212**, 378.