Studies on the Mechanism of Lanosterol 14 α -Demethylation

A REQUIREMENT FOR TWO DISTINCT TYPES OF MIXED-FUNCTION-OXIDASE SYSTEMS

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Carbon monoxide inhibited the removal of C-32 of dihydrolanosterol (I), but not of its metabolites 5 α -lanost-8-ene-3 β ,32-diol (II) and 3 β -hydroxy-5 α -lanost-8-en-32-al (III). It appears therefore that cytochrome *P*-450 is a component of the enzyme system required to initiate oxidation of the 14 α -methyl group, but not of that responsible for the subsequent oxidation steps required for elimination of C-32 as formic acid. Non-radioactive compounds (II) and (III), when added to cell-free systems actively converting dihydrolanosterol into cholesterol, inhibited 14 α -demethylation measured by the rate of formation of labelled cholesterol from dihydro[1,7,15,22,26,30-¹⁴C]lanosterol or of labelled formic acid from dihydro[32-¹⁴C]lanosterol. However, neither compound (II) nor compound (III) accumulated radioactive label under these conditions. These observations could be attributed partly to inhibition of the initial oxidation of the 14 α -methyl group by compounds (II) and (III).

It is now generally accepted that the 14 α -methyl carbon (C-32) of lanosterol is oxidatively removed as formic acid (Alexander *et al.*, 1972; Mitropoulos *et al.*, 1976; Akhtar *et al.*, 1978) and that an 8,14-diene (IX, Fig. 1) is the immediate sterol product of C-32 elimination (Alexander *et al.*, 1971, 1972; Akhtar *et al.*, 1972, 1978; Fiecchi *et al.*, 1972; Gibbons, 1974; Gibbons & Mitropoulos, 1975). The structures of the oxidized steroidal intermediates involved in this process, however, have not yet been clearly established. This is largely as a result of the continued failure to detect any such oxidized sterols during the biosynthesis of cholesterol from natural precursors. Experimental evidence currently available concerning the possible intermediacy of either 15-hydroxy-14 α -methyl sterols (IV) or 14 α -hydroxymethyl sterols (II) (each representative of one of the two alternative pathways shown in Fig. 1) therefore rests exclusively on the enzymic convertibility of these types of compound into cholesterol or into known cholesterol precursors (Fried *et al.*, 1968; Martin *et al.*, 1970; Alexander *et al.*, 1971, 1972; Spike *et al.*, 1974). The relative efficiency with which each of the proposed precursors that contain oxygen functions at either C-15 or C-32 is converted into cholesterol provides some indication of its status as a natural intermediate. It is essential, however, in making such comparisons that the oxidized sterols in question contain both an 8-ene bond and a 4,4-dimethyl grouping. Regardless of the exact oxidative sequence, the natural substrate of the liver demethylase enzymes contains both these features (Gautschi & Bloch, 1958; Gaylor *et al.*, 1966). The difficulty in synthesizing

these types of sterol has often compromised this requirement and it was not until more recently that a valid comparison was undertaken (Gibbons *et al.*, 1976). The very poor performance of the 15 α - and 15 β -hydroxylanost-8-enols (structure IV, Fig. 1) as cholesterol precursors observed in this latter study compared with 5 α -lanost-8-ene-3 β ,32-diol and 3 β -hydroxy-5 α -lanost-8-en-32-al (structures II and III, Fig. 1) diminished the claim of these former sterols as natural intermediates. Structures (II) and (III) remain, therefore, as the most likely candidates for obligatory status. As the first oxidative step after the formation of lanosterol utilizes cytochrome *P*-450 (Gibbons & Mitropoulos, 1973*a,b*), it would thus appear that the product of this cytochrome *P*-450-requiring step is the hydroxymethyl structure (II) (Fig. 1). The formation of the aldehyde (III) from structure (II), and its further metabolism to structure (IX) each require the participation of molecular oxygen and of NADPH (Alexander *et al.*, 1972; Akhtar *et al.*, 1978). The present work was undertaken to determine whether these steps that complete the demethylation utilized the same enzyme system, and in particular, cytochrome *P*-450, as the initial oxidative step. We have approached this problem by comparing the effect of CO on the metabolism of 5 α -lanost-8-en-3 β -ol (I, Fig. 1), a substrate of the initial and cytochrome *P*-450-requiring oxidase system, with its effect on the oxidative metabolism of its product structure (II) and of structure (III).

In a wider context, elucidation of the exact enzymic mechanism of 14 α -demethylation may have an important bearing on the regulation of cholesterol-

Fig. 1. Possible mechanisms involved in the removal of the 14 α -methyl group of lanosterol

genesis since the activity of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (EC 1.1.1.34), the proposed rate-limiting step in cholesterol synthesis (Rodwell *et al.*, 1976), is markedly influenced by small changes in the concentration of proposed intermediates of this process (Schroepfer *et al.*, 1977, 1978; Kandutsch *et al.*, 1978).

Experimental

Methods

The housing and treatment of rats, preparation of subcellular fractions of liver, emulsification of substrates, incubation procedures in the presence of air or artificial gas mixtures, isolation of the total lipid fraction, measurement of radioactivity, separation of the crude C_{27} sterol mixture (mainly cholesterol and 5 α -cholest-7-en-3 β -ol) by t.l.c. on silica gel H by using chloroform as the mobile phase (system 1) and acetylation of sterols have been described previously by Gibbons & Mitropoulos (1973*a,b*) and by Gibbons (1974). 3 β -Hydroxy-5 α -lanost-8-en-32-al had the same R_F value as the C_{27} sterol mixture in t.l.c. system 1. Where necessary, therefore, the aldehyde was separated from the C_{27} sterol mixture by acetylation followed by t.l.c. on silica gel H by using a mixture of chloroform and n-hexane (3:1, v/v; system 2). Separation of cholesterol from 5 α -cholest-7-en-3 β -ol was carried out by t.l.c. of the acetate derivatives on AgNO_3 -impregnated alumina by using a mixture of n-hexane and toluene (3:1, v/v) as the mobile phase (system 3). That the cholesterol and 5 α -cholest-7-en-3 β -ol fractions labelled from various substrates and isolated in this way were radiochemically pure has been shown by crystallization to constant specific radioactivity (Gibbons, 1974; Gibbons *et al.*, 1976). Assay of [^{14}C]formate was carried out as described by Mitropoulos *et al.* (1976).

Materials

The sources of the non-radioactive materials described in the present work have been reported by Gibbons & Mitropoulos (1973*a,b*) and Gibbons (1974). Dihydro[1,7,15,22,26,30- ^{14}C]lanosterol (hereafter referred to as dihydro[^{14}C]lanosterol) was prepared biosynthetically from [2- ^{14}C]mevalonate and extensively purified as described previously (Gibbons & Mitropoulos, 1973*a*). The preparation of 5 α -lanost-8-ene-3 β ,32-diol (II) and of 3 β -hydroxy-5 α -lanost-8-en-32-al (each either unlabelled or labelled with ^3H at C-2) has been previously reported (Gibbons *et al.*, 1976), as has the preparation of 4,4-dimethyl-5 α -[2- $^3\text{H}_2$]cholesta-8,14-dien-3 β -ol (Gibbons, 1974) and of dihydro[32- ^{14}C]lanosterol (Mitropoulos *et al.*, 1976).

Results and Discussion

Effect of CO on the metabolism of cholesterol precursors

5 α -[2-³H₂]Lanost-8-ene-3 β ,32-diol (II) and 3 β -hydroxy-5 α -[2-³H₂]lanost-8-en-32-al (III) were each incubated with an S₁₀ fraction of liver homogenate (i.e. the supernatant obtained after centrifugation at 10000g_{av.} for 20 min). This subcellular fraction contains the microsomal and soluble fractions of the cell. S₁₀ fractions prepared in this way contain a maximum of 1.5–2.0% of mitochondria as measured by the activity of the mitochondrial marker enzyme succinic dehydrogenase. Incubations were conducted in the presence of gas mixtures consisting of either N₂/O₂ (9:1, v/v) or CO/O₂ (9:1, v/v). The conversions of each of structures (II) and (III) into cholesterol and 5 α -cholest-7-en-3 β -ol were determined under these conditions. As controls, similar incubations were conducted by using the cholesterol precursors dihydro[¹⁴C]lanosterol (I), whose metabolism is known to be inhibited by CO, and 4,4-dimethyl-5 α -[2-³H₂]cholesta-8,14-dien-3 β -ol (IX) whose conversion into cholesterol is unaffected (Gibbons & Mitropoulos, 1975). The effects of CO on the incorporation of radioactivity from these compounds into cholesterol and 5 α -cholest-7-en-3 β -ol are shown in Table 1. As expected, CO potentially inhibited the conversion of dihydro[¹⁴C]lanosterol into C₂₇ sterols, an effect due to the inactivation of an enzyme that contains cytochrome P-450. This enzyme catalyses the first of the oxidative steps culminating in 14 α -demethylation, and probably gives rise to the hydroxymethyl sterol (II) (Fig. 1). The metabolism of 4,4-dimethyl-5 α -[2-³H₂]cholesta-8,14-dien-3 β -ol (IX, Fig. 1), which immediately results from removal of the 14 α -methyl (C-32) carbon, was unaffected in accordance with the proposition that a CO-sensitive cytochrome is not involved in any oxidative process of cholesterol biosynthesis other than the 14 α -demethylation of C₃₀ sterols (Gibbons & Mitropoulos, 1975). Under these conditions, metabolism of the oxygenated C-32 sterols (II) and (III) of the type proposed as natural cholesterol precursors (Alexander *et al.*, 1972; Akhtar *et al.*, 1977, 1978) was unaffected by CO. It is probable, therefore, that neither of the mixed-function-oxidase systems required to complete the demethylation process (II→III→IX; Fig. 1) contains a conventional CO-sensitive cytochrome P-450.

The insensitivity of these enzymes to CO was not due to the relatively low concentration of substrates in the incubation medium. This was shown by repeating the experiment at substrate concentration closer to those required for enzyme saturations (Gibbons *et al.*, 1976). Under these conditions, in the presence of CO, conversions into cholesterol of

Table 1. *Effect of CO on the metabolism of some cholesterol precursors*

Precursor	Amount incubated		Gas phase	Radioactivity (d.p.m.) in:		Relative effect* of CO on production of:	
	(d.p.m.)	(nmol)		Cholesterol	Cholest-7-enol	Cholesterol	Cholest-7-enol
Dihydrolanosterol	0.114 × 10 ⁶	23.5	N ₂ /O ₂	2500	4100		
			CO/O ₂	1200	1600	48.0	39.0
4,4-Dimethyl-5 α -cholesta-8,14-dienol	0.337 × 10 ⁶	24.3	N ₂ /O ₂	11700	33100		
			CO/O ₂	12100	30600	103.4	92.5
5 α -Lanost-8-ene-3 β ,32-diol	1.261 × 10 ⁶	90.1	N ₂ /O ₂	32500	71400		
			CO/O ₂	32000	58700	98.5	82.2
3 β -Hydroxy-5 α -lanost-8-en-32-al	0.810 × 10 ⁶	56.6	N ₂ /O ₂	34200	45300		
			CO/O ₂	35100	54500	102.6	120.3

* Amount of sterol formed in the presence of N₂/O₂ = 100.

dihydro[^{14}C]lanosterol present at a concentration of $93.5\mu\text{M}$ and 4,4-dimethyl-5 α -[2- $^3\text{H}_2$]cholesta-8,14-dien-3 β -ol at a concentration of $77.7\mu\text{M}$ were 53.6% and 107.5% respectively of the corresponding conversions observed in the presence of N_2/O_2 . In the same experiment with radioactively labelled structures (II) and (III) as substrates at concentrations of $144\mu\text{M}$ and $108.6\mu\text{M}$ respectively, CO again had no effect on the formation of cholesterol, the observed conversions being 104.9% and 112.8% respectively of those observed in the presence of N_2/O_2 .

The possibility remained, however, that inhibition by CO occurred only during shorter incubation periods in which the rate of C_{27} sterol formation was constant with respect to time. Consequently, the effect of length of incubation time on the incorporation of radioactivity from labelled structures (II) and (III) into C_{27} sterols was investigated. The labelled sterol precursors were each incubated for different periods of time in the presence of a gas phase of N_2/O_2 (9:1, v/v). Cholesterol and 5 α -cholest-7-en-3 β -ol were isolated as their acetate derivatives, and their radioactivities were determined. For a period of 20–30 min the C_{27} sterols became labelled at a constant, or slightly accelerating, rate (Fig. 2). Simultaneous incubations were conducted with each substrate for periods of 15 and 20 min in the presence of CO/O_2 (9:1, v/v). In no case was there any decrease in the incorporation of radioactivity into either cholesterol or 5 α -cholest-7-en-3 β -ol when compared with the corresponding incubations conducted for the same periods of time in the presence of N_2/O_2 (9:1, v/v).

Effect of C-32-oxygenated sterols on the metabolism of dihydro[^{14}C]lanosterol

Certain precursors of cholesterol are capable of simultaneously accumulating or trapping radioactive label and diminishing the incorporation of radioactivity into cholesterol when they are added in relatively large quantities during incubations in which cholesterol is being actively synthesized from established radiochemically labelled precursors. This information has provided compelling evidence for the natural intermediacy of mevalonate (Knauss *et al.*, 1959), squalene (Langdon & Bloch, 1953), lanosterol (Clayton & Bloch, 1956) and of 4,4-dimethyl-5 α -cholesta-8,14-dien-3 β -ol (Fiechi *et al.*, 1972). The failure of a compound to behave in this ideal way, however, does not exclude it from obligatory intermediacy, since the negative result may have been due to a wide range of other factors (see below).

We have carried out an experiment such as this to ascertain whether the 32-hydroxymethyl sterol (II) or the 32-aldehyde (III) or both are capable of trapping radioactivity during cholesterol biosyn-

Fig. 2. Time course of C_{27} sterol formation from 5 α -[2- $^3\text{H}_2$]lanost-8-ene-3 β ,32-diol and 3 β -hydroxy-5 α -[2- $^3\text{H}_2$]lanost-8-en-32-al

(a) 5 α -[2- $^3\text{H}_2$]lanost-8-ene-3 β ,32-diol (1.112×10^6 d.p.m., 79.5 nmol) or (b) 3 β -hydroxy-5 α -[2- $^3\text{H}_2$]lanost-8-en-32-al (1.110×10^6 d.p.m., 77.6 nmol) were each suspended with the aid of Tween 80 (0.6 mg) in potassium phosphate buffer (0.1 ml, pH 7.4) and incubated with an S_{10} fraction (0.4 ml, 13.0 mg of protein) of rat liver homogenate for the periods of time indicated. In all cases the gas phase consisted of a mixture of N_2/O_2 (9:1, v/v). The final volume of each incubation mixture was 0.6 ml. Other details are given in the legend to Table 1. Symbols: ■, incorporation into cholesterol; ▲, incorporation into 5 α -cholest-7-en-3 β -ol; ●, incorporation into total C_{27} sterols.

thesis from labelled dihydrolanosterol, and of decreasing the amount of radioactively labelled cholesterol produced. The results of this experiment

are given in Table 2, which shows that, whereas structure (II) resulted in only a moderate inhibition of dihydro[^{14}C]lanosterol conversion into cholesterol, structure (III) was a better inhibitor. However, extensive chromatographic purification of the fraction containing structure (III) from these incubations revealed that the inhibition of labelled cholesterol synthesis was not accompanied by a significant accumulation of radioactivity in structure (III), which contained only 420 d.p.m. (0.21% of the incubated radioactivity). As the amount of radioactivity associated with this fraction declined appreciably during successive chromatographic steps, it was considered unlikely that even this small amount represented radiochemically pure material.

Effect of C-32-oxygenated sterols on the rate of release of [^{14}C]formic acid from dihydro[32- ^{14}C]lanosterol

A similar experiment was carried out by using the microsomal fraction alone as the source of enzymes with dihydro[32- ^{14}C]lanosterol as the labelled substrate. In this case, the rate of labelled formic acid formation was measured in the presence and absence of unlabelled structures (II) and (III). The use of this labelled substrate allows a direct measurement of 14 α -demethylase activity (Mitropoulos *et al.*, 1976). Incubation of dihydro[32- ^{14}C]lanosterol in the absence of structures (II) and (III) for different periods of time and with different concentrations of microsomal protein showed that the rate of release of labelled formic acid was constant for up to 30 min at concentrations of microsomal protein ranging from 0–2 mg/ml of incubation medium (Fig. 3). To determine the effects of unlabelled structures (II) and (III) on the rate of [^{14}C]formic acid release from dihydro[32- ^{14}C]lanosterol, each of the unlabelled sterols were

incubated at two different concentrations in the presence of microsomal fraction (1.9 mg of protein/ml) for 30 min.

To compensate for possible non-specific effects, such as differences in accessibility of the labelled substrate to the enzyme active site, introduced by the presence of a larger quantity of non-radioactive steroid, parallel incubations were conducted in the presence of similar quantities of cholesterol. These were used as control incubations against which the effects of structures (II) and (III) were measured. The results are shown in Table 3. Although the presence of cholesterol had little or no effect, both structure (II) and structure (III) were inhibitory towards 14 α -demethylation, the latter having by far the greatest effect. Inhibition was not accompanied by a compensatory increase in the radioactivities of the added C-32-oxygenated sterols. Although in some cases there appeared to be a slight increase in the radioactivity associated with these compounds in the incubations conducted in their presence, the very low amounts of radioactivity involved precluded any further attempts at identification. The fact that the radioactivity of each of these fractions decreased substantially after successive chromatographic steps suggested that very little, if any, label was radiochemically identical with either structure (II) or structure (III). In another, similar experiment, non-radioactive structure (II) present at concentrations of 66 $\mu\text{g/ml}$ and 165 $\mu\text{g/ml}$ gave rise to decreases of 66.2% and 80.1% respectively in the rate of release of [^{14}C]formic acid. Identical concentrations of structure (III) resulted in decreases of 83.7% and 90.0% respectively. In agreement with the results with dihydro[^{14}C]lanosterol, the presence of the C-32-oxygenated sterols resulted in a decreased rate of metabolism of dihydro[32- ^{14}C]lanosterol, as measured by the quantity of this material recovered at the end of these incubations (Table 3). This suggests that these compounds exert their inhibitory

Table 2. *Effect of C-32-oxygenated sterols on the rate of formation of C_{27} sterols from dihydro[^{14}C]lanosterol* Dihydro[^{14}C]lanosterol (199 000 d.p.m.; 11 624 d.p.m./nmol) was emulsified in 0.1 M-potassium phosphate buffer (0.1 ml, pH 7.4) in the presence of Tween 80 (1.0 mg) and added to an S_{10} fraction of rat liver homogenate (2.0 ml). 5 α -Lanost-8-ene-3 β ,32-diol (500 μg) or 3 β -hydroxy-5 α -lanost-8-en-32-al (500 μg) each of which had been emulsified in a similar buffer (0.1 ml) containing Tween 80 (4.0 mg) was then added. To incubations conducted in the absence of the unlabelled sterols was added buffer (0.1 ml) containing Tween 80 (4.0 mg) alone. The concentrations of cofactors and other constituents are given in the legend to Table 1. The flasks (total volume of contents, 3.0 ml) were incubated at 37°C for 45 min. After extraction of the total lipid material, fractions containing the radioactive sterols were obtained by t.l.c.

Sterol added	Radioactivity (% of that incubated) associated with:		
	Cholesterol	5 α -Cholest-7-en-3 β -ol	Dihydrolanosterol
—	1.03	1.37	60.80
5 α -Lanost-8-ene-3 β ,32-diol	0.66	0.99	67.63
3 β -Hydroxy-5 α -lanost-8-en-32-al	0.42	0.13	73.17

Fig. 3. Kinetics of $[^{14}\text{C}]$ formic acid release from dihydro- $[32\text{-}^{14}\text{C}]$ lanosterol

(a) Dihydro $[32\text{-}^{14}\text{C}]$ lanosterol (56700 d.p.m., 18.7 nmol) was emulsified in the presence of Tween 80 and incubated with rat liver microsomal fraction (1.95 mg of protein) for various periods of time. (b) Dihydro $[32\text{-}^{14}\text{C}]$ lanosterol (56700 d.p.m., 18.7 nmol) was emulsified in the same way as above and incubated for 30 min in the presence of increasing concentrations of microsomal protein. In all cases the final volume of the incubation mixture was 1.0 ml. Other experimental details are given in the Legends to Tables 1 and 3.

Table 3. Effect of C-32-oxygenated sterols on the rate of $[^{14}\text{C}]$ formic acid production from dihydro $[32\text{-}^{14}\text{C}]$ lanosterol. Dihydro $[32\text{-}^{14}\text{C}]$ lanosterol (240000 d.p.m., 3030 d.p.m./nmol) was emulsified with Tween 80 and added to a suspension of rat liver microsomal fraction (0.6 ml; 12.9 mg of protein/ml) containing cofactors and potassium phosphate buffer (pH 7.4) as described previously (Mitropoulos *et al.*, 1976) in a total volume of 4.0 ml. The sterols were added as an emulsion in potassium phosphate buffer (pH 7.4) containing Tween 80. The final concentration of detergent was 0.45 mg/ml in those incubations containing the lower concentration of sterol and 0.8 mg/ml in those containing the higher concentrations. Incubations were conducted for 30 min at 37°C and were terminated by the addition of chloroform/methanol (2:1, v/v). Dihydro $[32\text{-}^{14}\text{C}]$ lanosterol was isolated by t.l.c. and $[^{14}\text{C}]$ formic acid was assayed as described previously (Mitropoulos *et al.*, 1976). For other details, the legend to Table 1 should be consulted.

Sterol added	Concentration ($\mu\text{g}/\text{ml}$)	Radioactivity (% of that incubated) associated with				Decrease in rate of formic acid (%) release*
		Dihydrolanosterol	(II)	(III)	Formic acid	
Cholesterol	25	89.1	0.08	0.23	6.6	
	50	91.6	0.13	0.41	5.4	
5 α -Lanost-8-ene-3 β ,32-diol (II)	25	92.8	0.12	0.35	5.5	16.7
	50	91.5	0.26	0.39	3.6	33.3
3 β -Hydroxy-5 α -lanost-8-en-32-al (III)	25	95.5	0.49	0.55	0.6	91
	50	94.8	0.62	0.30	0.1	100

* This refers to the rate compared with that observed at the corresponding concentration of cholesterol.

effect by interfering with the initial oxidation of the 14 α -methyl group of dihydrolanosterol. The presence of these compounds in the culture medium also resulted in the accumulation of labelled lanosterol during cholesterol biosynthesis from $[1\text{-}^{14}\text{C}]$ acetate in Chinese hamster lung cells (W. Cavenee, A. A. Kandutsch, H. Chen & G. F. Gibbons, unpublished work), suggesting that the initial oxidation of the 14 α -methyl group was impaired.

These observations raise the possibility that dihydrolanosterol, 5 α -lanost-8-ene-3 β ,32-diol and 3 β -hydroxy-5 α -lanost-8-en-32-al compete for some component of a microsomal electron-transport

chain, but not cytochrome P-450, which is common to both the initial and subsequent oxidative stages of 14 α -demethylation. This component may be a flavoprotein, since these have been shown to have a rôle in other mixed-function-oxidase systems involved in cholesterol biosynthesis (Ono & Bloch, 1975). Despite this similarity, a major difference in the composition of the enzyme systems responsible for the initial, as opposed to the subsequent, oxidation is the presence, in the former, of cytochrome P-450. A recent suggestion (Akhtar *et al.*, 1978) that the same enzyme system catalyses the initial oxidation of the methyl group and the subsequent oxidation of the

resulting alcohol to the aldehyde cannot therefore be substantiated experimentally.

The inability of structures (II) and (III) to accumulate radioactivity therefore may be attributed, at least partly, to their inhibitory effect on the metabolism of their precursor, dihydrolanosterol. This should not rule out the possibility that other factors influence the outcome of experiments with complex multienzyme systems such as this, in which it is difficult to obtain valid kinetic information. For instance, the lack of any detectable radioactivity associated with structure (II), even though its presence did not result in complete inhibition of dihydrolanosterol metabolism (Table 3), may be ascribed to the irreversible formation of enzyme complexes of these oxygenated sterols. These complexes, formed from the added C-32-oxygenated sterols, would be identical with those formed biosynthetically from dihydrolanosterol and would not dissociate appreciably to the free unbound oxysterol. The continued failure to detect the free products of initial oxidation, not only during 14 α -demethylation, but also during the elimination of the 4 α - and 4 β -methyl groups during cholesterol biosynthesis, suggests that such compounds are formed and metabolized only as the enzyme complexes or other protein-bound intermediates described above.

The exact chemical mechanism by which the aldehyde is oxidatively metabolized to the diene (III $\rightarrow$ IX; Fig. 1) and formic acid is largely a matter of conjecture, although further oxidation to give an enzyme-bound complex (VII) has been proposed. Loss of formic acid could then arise either by a concerted mechanism (VII $\rightarrow$ IX; Fig. 1) or by a Baeyer-Villiger-type rearrangement of the oxidized complex to give the formate ester followed by cleavage of the C-14-oxygen bond (VII $\rightarrow$ VIII $\rightarrow$ IX; Fig. 1) (Akhtar *et al.*, 1978). Alternatively the third oxygen atom may never

become incorporated into the sterol molecule, the C-14-C-32 bond being cleaved with concomitant oxidation of the aldehyde and elimination of the 15 α -hydrogen atom to give formic acid and introduction of the C-14-C-15 double bond. In this case the acceptor of the formyl group may be an electron-deficient oxygen function generated by heterolytic fission of an enzyme-bound peroxide linkage (Fig. 4).

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Fig. 4. Possible mechanism for the further metabolism of 3 β -hydroxy-5 α -lanost-8-en-32-al involving an electron-deficient oxygen function