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The Substrate Activation in Some Pyridine Nucleotide Linked Enzymic Reactions

The Conversion of Desmosterol into Cholesterol

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The conversion of desmosterol into cholesterol was carried out in the presence of either tritiated water or [4-$^3H_2$]NADPH. The side chain fragment consisting of carbon atoms 23–27 of cholesterol was obtained through the combination of biological and chemical techniques. Selective degradation of the fragment led to the conclusion that in the saturation of the 24,25-double bond of desmosterol a hydrogen atom from the medium is added to C-24 and another from the 4-position of NADPH to C-25. These results in conjunction with similar studies previously reported on the saturation of other C=C in steroid biosynthesis are interpreted in terms of a general mechanism. It is suggested that the first crucial event in the pyridine nucleotide linked reduction is the activation of the substrate through protonation to give an electron deficient species which in the next step is neutralized by the addition of a hydride from NAD(P)H to furnish the product.

Pyridine nucleotides have been extensively implicated in the reduction of groups such as C=O, C=N and C=C and many pyridine nucleotide linked enzymes have been purified and subjected to extensive kinetic analysis. Much valuable data has been obtained concerning the order of binding of substrates (for review see [1]) but these studies have yielded little information concerning the actual mechanism and sequence of bond formation.

We envisaged that the orientation of addition of a proton from the medium and a hydride ion from NADPH to olefinic linkages may give information concerning the precise mechanism of C=C reduction reactions. These mechanistic conclusions should have some bearing on the mechanism of substrate activation in other pyridine nucleotide linked reductions involving C=O and C=N.

In this paper we study the reduction of the 24–25 double bond during the conversion of cholesta-5, 24-dien-3β-ol (I) (desmosterol) into cholesterol (II) and discuss the results obtained from this study and other work we have reported on the reduction of sterol double bonds at 7–8 and 14–15 [2,3]. A preliminary report of this work has been published [4].

EXPERIMENTAL PROCEDURE

Materials

Cholesta-5, 24-dien-3β-ol (I) was prepared from 3β-acetoxy-26-norcholest-5-en-25-one by the method of Dauben and Bradlow [5]. [4-$^3H_2$]NADPH (2×10^6 counts $\times$ min$^{-1}\times\mu$mol^{-1}) was prepared by the method of Wilton *et al.* [2]. All other materials were obtained as described by Wilton *et al.* [2].

Incubation Procedure and Isolation and Purification of Cholesterol

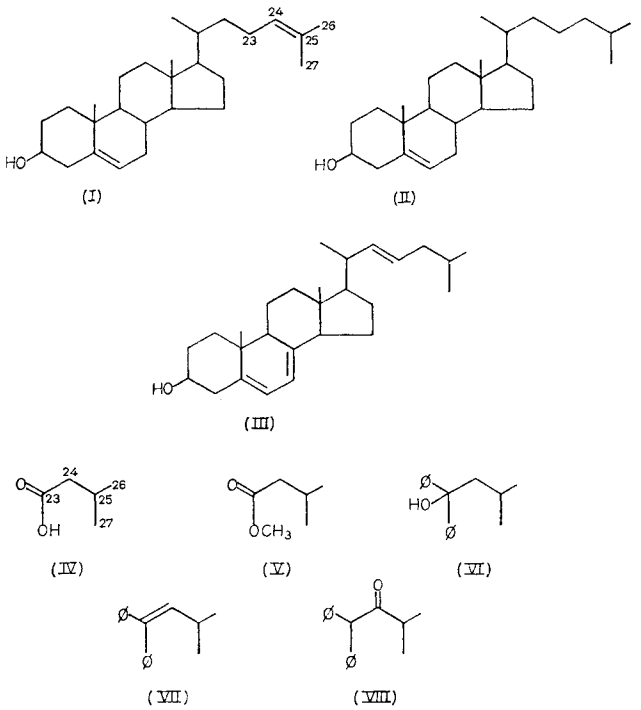
The methods used have been described previously [2,10].

Incubation of Cholesterol with Tetrahymena pyriformis *and the Isolation of Cholesta-5,7,22-triene-3β-ol (III)*

The incubation of cholesterol with *Tetrahymena pyriformis* and the isolation of (III) have been described previously [13]. The yield of (III) from cholesterol in various experiments ranged from 40–60%.

Oxidation of Cholesta-5,7,22-trien-3β-ol (III) and the Extraction of Isovaleric Acid (IV)

Cholesta-5,7,22-trien-3β-ol (20–25 mg) was dissolved in *tert*-butanol (90 ml) and to this was added K_2CO_3 (9 ml; 50 mM) and a mixture (50 ml) of $KMnO_4$ (3 mM) and $NaIO_4$ (97 mM). The mixture was maintained at 37 °C overnight in a gently shaking stoppered flask. Isovaleric acid (5 ml) was then added, and the reaction mixture was acidified with H_2SO_4 and decolourised by the dropwise addition of sodium metabisulphite. The solution was extracted with di-



Scheme 1. *Structures of desmosterol, cholesterol and derivatives.* In order to allow direct comparison the steroid side chain numbering has been retained for the compounds (IV) ← (VIII)

ethyl ether (4 × 20 ml), neutralized with methanolic KOH and evaporated to dryness. The residue was acidified with diluted H_2SO_4 and extracted with ether. The organic layer after being dried (Na_2SO_4) was distilled to yield isovaleric acid (b.p. 178—180 °C).

Preparation of Methyl Isovalerate (V)

To the radioactive iso-valeric acid from the above experiment was added an excess of diazomethane in ether. Distillation yielded methyl isovalerate (V) (3.7 g; b.p. 116—117). The overall radiochemical yield from cholesterol to methyl isovalerate was about 25%.

Base Equilibration of Methyl Isovalerate (V)

Sodium metal (3 g) was dissolved in dry methanol (20 ml) and this was then stirred with gentle heating under reflux. Purified nitrogen was passed over the solution until the atmosphere was completely oxygen free. Methyl isovalerate (1 ml) was then added and both stirring and heating were continued under an atmosphere of nitrogen for 18 h. At the end of this time the solution was extracted (4 × 20 ml) with light petroleum (b.p. less than 40 °C). The pooled extracts were washed with water and saturated NaCl solution and then dried with anhydrous sodium sulphate. The solution was then reduced in volume to 10 ml and the methyl isovalerate obtained by distillation. It was purified by redistillation.

Preparation of Phenyl Magnesium Bromide

Clean, dried magnesium turnings (2 g) were placed in a 100 ml round bottom flask fitted with a condenser and drying tube and the system was flushed with nitrogen. A solution of bromobenzene (9 ml) in sodium dried ether (15 ml) was added and the mixture was warmed in a 50 °C bath to initiate the reaction. Once the reaction had started, 25 ml more dried ether was added and the reaction was left to go to completion (about 15 min) under an atmosphere of nitrogen.

Preparation of 1,1-Diphenyl-3-methylbutan-1-ol (VI)

Methyl isovalerate (8.5 ml) was dissolved in sodium dried ether (15 ml) and this was slowly added to the rapidly stirring phenyl magnesium bromide solution. The mixture was then heated under reflux for 2 h, after which time an equal volume of 10% H_2SO_4 was added, and the aqueous fraction was separated and extracted with diethyl ether. The pooled ether extract was washed with water and saturated NaCl solution before being dried with anhydrous sodium sulphate. The yellow oily product obtained on evaporation of solvent was purified by distillation under reduced pressure (115—125 °C, 0.7 mm Hg) to yield 1,1-diphenyl-3-methylbutan-1-ol (VI) as a colourless oil (8.3 g).

Preparation of 1,1-Diphenyl-3-methylbut-1-ene (VII)

1,1-Diphenyl-3-methylbutan-1-ol (8.3 g) was dissolved in diethyl ether (40 ml) and to this was added 50% H_2SO_4 (20 ml). The mixture was heated under reflux with rapid stirring for one hour before the aqueous layer was separated and extracted with diethyl ether (20 ml). The pooled ether extracts were worked up as described above. The slightly yellow oil was purified by distillation under reduced pressure (115—120 °C, 0.4 mm Hg) to yield 1,1-diphenyl-3-methylbut-1-ene (VII) as a colourless oil (7.8 g); λ_{max} 250 nm (ε = 14000) (Found: C, 91.6; H, 8.3. $C_{17}H_{18}$ requires C, 91.8; H, 8.2).

Preparation of 1,1-Diphenyl-3-methylbutan-2-one (VIII)

A solution of 1,1-diphenyl-3-methylbut-1-ene (VII) (1.0 g) and *m*-chloroperbenzoic acid (1.2 g) in chloroform (30 ml) was left at room temperature over night. After the addition of water the organic layer was washed several times with 10% aqueous KOH, dried (Na_2SO_4) and evaporated to dryness. The residue was dissolved in dioxan (20 ml) containing conc. HCl (10 ml) and heated at 100 °C for 2 h. After

the addition of water the reaction mixture was extracted with ether. The organic layer was washed with aqueous saturated $NaHCO_3$, dried (Na_2SO_4) and evaporated to dryness. The residue was applied to four preparative layer plates which were run in ether—light petroleum (b.p. 60—80 °C) (100:5, v/v). The band with R_F 0.4 was removed, eluted with ether to give after crystallization from light petroleum (b.p. 60—80 °C) 1,1-diphenyl-3-methylbutan-2-one (VIII) 200 mg, m.p. 68—71 °C, γ_{max}^{Nujol} 1710 cm^{-1}; molecular weight by mass spectrometry 238 (Found: C, 85.5; H, 7.7. $C_{17}H_{18}$ requires C, 85.7; H, 7.6%).

The ^{14}C specific radioactivities of compounds (V), (VII) and (VIII) were 902, 928 and 918 counts $\times$ min^{-1} $\times$ $mmole^{-1}$, respectively.

Measurement of Radioactivity

Radioactivity was measured as described previously [13].

RESULTS

Conversion of Desmosterol (I) to Cholesterol (II) in the Presence of Either [4-$^3H_2$]NADPH or Tritiated Water

Desmosterol (I; 1 mg) prepared by the method of Dauben and Bradlow [5] from 26-norcholesta-5-en-3β-ol-25-one acetate, was incubated with a 105000 $\times$ g microsomal preparation of rat liver homogenate in the presence of [4-$^3H_2$]NADPH (2 μmol; 2×10^6 counts $\times$ min^{-1} $\times$ μmol^{-1}) to give cholesterol (II; 3.36×10^5 counts/min) which was purified through its dibromide derivative [6]. The results of several such experiments were pooled to give 6.06×10^6 counts/min of purified cholesterol.

In an alternative experiment, desmosterol (I; 1 mg) was incubated with a 105000 $\times$ g microsomal fraction of rat liver homogenate in the presence of tritiated water (the specific activity of the medium was 1.7 mCi/mg atom of hydrogen) and non-radioactive NADP (1.5 μmol) together with an NADPH generating system. The biosynthesised cholesterol, purified as above, contained 4.2×10^5 counts/min. A control experiment which lacked the substrate incorporated less than 1% of the activity of the experimental flask.

Analysis of the Cholesterol (II) Formed from Desmosterol (I) in the Presence of Tritiated Water

The biosynthetic cholesterol was mixed with [26,27-^{14}C]cholesterol and the resulting material ($^3H/^{14}C$ ratio 1.00) was incubated with whole cells of *Tetrahymena pyriformis* [7]. The isolated 5α-cholesta-5,7,22-trien-3β-ol (III) derived from the cholesterol was subjected to side chain cleavage [7] between the 22—23 double bond with potassium carbonate-sodium periodate-potassium permanganate to give isovaleric acid (IV). The isovaleric acid was extracted and converted into its methyl ester (V) ($^3H/^{14}C$ ratio 0.82) which had lost about 18% of the tritium. This loss may be attributed to the base catalysed equilibration of the α-hydrogens of the isovaleraldehyde which is probably a transistory intermediate in the oxidation of the sterol side chain to isovaleric acid (IV). The methyl iso-valerate (V) was then heated under reflux for 18 h in a mixture of sodium methoxide in dry methanol to allow the exchange of the α-hydrogens of the ester (C-24 of cholesterol) with the protons of the medium. The recovered ester had lost all its tritium ($^3H/^{14}C$ ratio 0.03). These results, which are summarised in Table 1, conclusively show that in the conversion of desmosterol (I) into cholesterol (II) in the presence of tritiated water the radioactivity that is incorporated in the fragment containing carbon atoms 23, 24, 25, 26 and 27 is exclusively located at C-24.

Table 1. *Analysis of cholesterol (II) biosynthesised from desmosterol (I) in the presence of tritiated water*

Compound	$^3H/^{14}C$ ratio	Tritium lost
		%
Cholesterol (II)	1.00	0
Methyl isovalerate (V)	0.82	18
Methyl isovalerate (V) base equilibrated	0.03	97

Analysis of the Cholesterol (II) Biosynthesised from Desmosterol (I) in the Presence of [4-$^3H_2$]NADPH

The biosynthetic cholesterol (II) was mixed with [26,27-^{14}C]cholesterol and the resulting material ($^3H/^{14}C$ ratio 1.00) was treated as described above to yield methyl isovalerate (V) ($^3H/^{14}C$ ratio 0.92). This ester was treated as above with base, and after extraction was found to have retained all its tritium ($^3H/^{14}C$ ratio 0.91). This analytical sequence was repeated on a second sample of cholesterol and this sample also lost no tritium on being taken through the same degradative sequence (see Table 2) Under these basic conditions a synthetic sample of methyl isovalerate labelled with tritium in the α-position lost 90% of its radioactivity.

The complete retention of tritium in these experiments suggests that although the hydrogen atom obtained from NADPH was present in the fraction containing carbon atoms 23, 24, 25, 26 and 27 it was not present at C-24. Once can deduce therefore that it must be located at C-25 and in order to confirm this deduction a further series of experiments were performed.

Biosynthetic cholesterol was mixed with [26,27-^{14}C]cholesterol and a sample of the resulting material

Table 2. *Analysis of cholesterol (II) biosynthesised from desmosterol (I) in the presence of [4-$^3H_2$]NADPH*

Compound	$^3H/^{14}C$ ratio		Tritium lost
	Expt 1	Expt 2	
			%
Cholesterol (II)	1.00	1.00	0
Cholesta-5,7,22-trien-3β-ol (III)	—	0.98	2
Methyl isovalerate (V)	0.92	0.97	5
Methyl isovalerate (V) base equilibrated	0.91	0.98	5

Table 3. *Final analysis of the cholesterol (II) biosynthesised from desmosterol (I) in the presence of [4-$^3H_2$]NADPH*

Compound	$^3H/^{14}C$ ratio	Tritium lost
		%
Cholesterol (II)	1.00	0
Cholesta-5,7,22-trien-3β-ol (III)	0.97	3
Methyl isovalerate (V)	1.01	0
1,1-Diphenyl-3-methylbutan-1-ol (VI)	0.98	2
1,1-Diphenyl-3-methylbut-1-ene (VII)	0.97	3
1,1-Diphenyl-3-methylbutan-2-one (VIII)	0.04	96

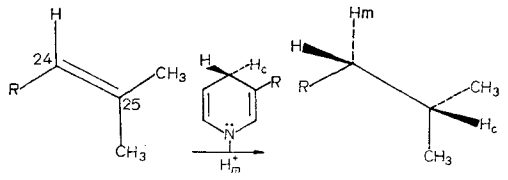
($^3H/^{14}C$ ratio 1.00) was subjected to the same side chain cleavage and methylation as above to yield methyl isovalerate (V) ($^3H/^{14}C$ ratio 1.01). The methyl isovalerate was converted to 1,1-diphenyl-3-methylbutan-1-ol (VI) and then to 1,1-diphenyl-2-methyl-but-1-ene (VII) ($^3H/^{14}C$ ratio 0.95) without further loss of tritium (see Table 3). This confirms, as previously established, that no tritium was present at C-24 of cholesterol, since half of the hydrogens previously present at this position are lost in the formation of the double bond of (VII).

Reaction of 1,1-diphenyl-3-methyl-but-1-ene (VII) with *m*-chloroperbenzoic acid followed by treatment of the reaction mixture with conc. HCl in dioxan for 2 h at 100 °C gave after chromatography the ketone (VIII) in about 25% yield. The latter compound, 1,1-diphenyl-3-methyl-butan-2-one (VIII), had the expected elemental composition, molecular weight and infrared spectrum and was completely devoid of the tritium radioactivity. The loss of tritium in the conversion (VII) → (VIII) can only be rationalised if the radioactive hydrogen was originally located at C-3 (corresponding to C-25 of cholesterol) of the ketone (VIII) and was removed by the acid catalysed exchange of its α-hydrogen atom with the protons of the medium under the vigorous conditions of the experiment.

These results confirm that in the conversion of desmosterol (I) into cholesterol (II) in the presence of [4-$^3H_2$]NADPH the radioactivity that is incorporated into the fragment containing carbo atoms 23, 24, 25, 26 and 27 is exclusively present at C-25.

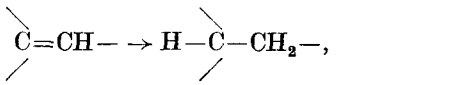
Thus in the reduction of the 24—25 double bond of desmosterol (I) a hydrogen atom from the medium is incorporated at C-24 and another from the 4-position of NADPH is transferred to C-25.

Recent elegant work in Caspi's laboratory [8,9] established that the newly formed C—H bonds at C-24 and C-25 are introduced in a *trans* configuration while it is the 4*B* hydrogen which is transferred from NADPH [10]. Thus the complete orientation of addition of hydrogens to the 24—25 double bond has been established and is summarised as shown below.



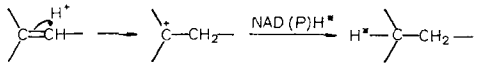
DISCUSSION

The saturation of a hypothetical unsymmetrical olefinic linkage



may occur by one of at least three mechanisms.

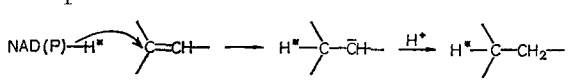
Mechanism 1. In the first mechanism the crucial event is the electrophilic addition of a proton to the substrate with the consequent formation of the carbonium ion intermediate (a). This intermediate is then neutralised by the delivery of a hydride ion from the reduced pyridine nucleotide to give the product. The reaction occurring through this mechanism will result in the Markownikoff mode of addition.



(a)

Mechanism 1

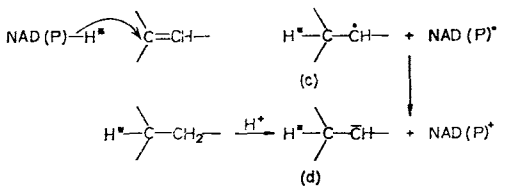
Mechanism 2. Mechanism 2 involves the initial nucleophilic attack by the reduced coenzyme on the substrate leading to the formation of a negatively charged species (b) which is then neutralized by the addition of a proton. In organic chemistry the addition to olefinic double bonds not activated by electron withdrawing groups seldom occurs by this type of mechanism. Such a process, however, when observed requires the participation of a strong nucleophile.



(b)

Mechanism 2

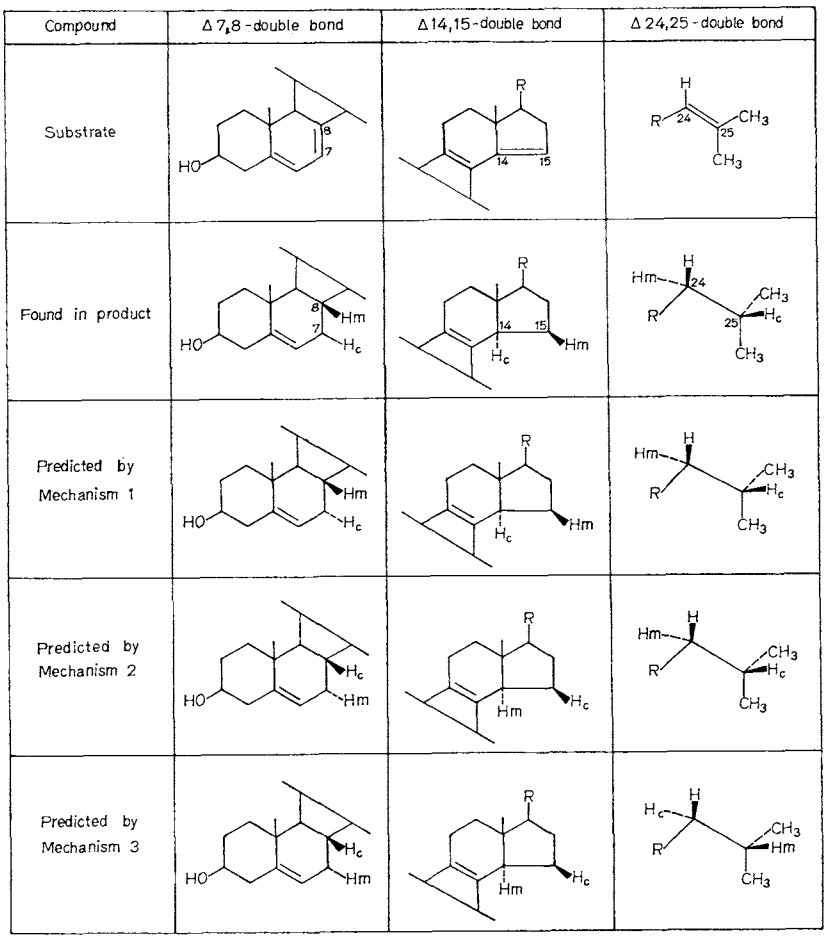
Mechanism 3. In mechanism 3 the initial event is the addition of a hydrogen atom (H·) from the reduced coenzyme giving a free radical intermediate (c) which then removes the spare electron on the NAD(P)+. The carbanion is then neutralised by addition of a proton as in mechanism 2. The need to consider a radical mechanism for the reaction is necessitated by several observations on model chemical systems which suggest that the 4-position of dihydropyridines can participate in homolytic as well as heterolytic reactions [11].



Mechanism 3

Isotope studies carried out on the NADPH-catalysed reduction of the steroidal double bonds at $\Delta^{7,8}$ [2] $\Delta^{14,15}$ [3] and $\Delta^{24,25}$ (see above) have shown that the positions of addition of a hydrogen H_m from the medium and a hydrogen H_c from NADPH is as seen in Scheme 2. If the orientation of addition of hydrogens to these three double bonds is now predicted for each of the three mechanisms described above and the results compared to those obtained experimentally, it should be possible to rationalise the reaction mechanisms involved.

The location of hydrogen atoms derived from NADPH and the medium predicted by the three mechanisms (Scheme 2) can be deduced by assuming that in each case the initial reaction results in the formation of the most favoured electronic species which is subsequently neutralised in the appropriate manner. It can be seen that in every instance the orientation of addition determined experimentally



Scheme 2. *Orientation of addition of hydrogens to sterol double bonds at $\Delta^{7,8}$, $\Delta^{14,15}$ and $\Delta^{24,25}$ found experimentally and as would have been predicted by mechanisms 1, 2 and 3.* H_m is the hydrogen from the medium; H_c is the hydrogen from the cofactor

(Scheme 2) corresponds to the positions predicted by Mechanism 1. This involves the electrophilic addition of a proton to the more electron rich terminal of the double bond, which in these cases would be C-8, C-15 and C-24 leading to the formation of the more favoured carbonium ions at C-7, C-14 and C-25 (Markownikoff addition). The subsequent addition of a hydride ion from NADPH to neutralize these carbonium ions would result in the overall orientation as shown in Scheme 2.

One may argue that in pyridine nucleotide dependent reduction reactions the hydrogen transfer from nucleotide to substrate occurs within a rigid ternary complex of substrate, nucleotide and enzyme. The position occupied by the hydrogen atoms in the product therefore depends on the orientation in which the nucleotide and substrate are held on the enzyme surface and not on the electronic distribution within the bond to be reduced. However in the present work we have seen that the reduction of three double bonds which possess very different steric and electronic environments displayed one common feature, that is in each case the hydrogen from the medium was added to the more electron rich terminus of the double bond and the hydrogen from the 4 position of NADPH to the more electron deficient carbon atom.

These results, readily explicable in terms of Mechanism 1, highlight the requirement for activation of the substrate by enzymic protonation prior to hydride transfer from the coenzyme in the pyridine nucleotide linked reduction of C=C. That a similar sequence of events may operate in other pyridine nucleotide linked reductions has been considered [12].

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