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CHOLESTEROL OXIDASE

FURTHER STUDIES OF SUBSTRATE SPECIFICITY IN RELATION TO THE ANALYTICAL CHARACTERISATION OF STEROIDS

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SUMMARY

The substrate specificity of cholesterol oxidase has been further examined with respect to the size and shape of the steroid 17β-side-chain and oxygenation of the nucleus. The relatively unreactive 17-keto-Δ^5-3β-hydroxysteroids were converted to satisfactory substrates by formation of the isopentyl- or benzyloximes. The results obtained have been used to illustrate further uses of the enzyme in the gas-phase analytical characterisation and selective oxidation of steroids. Oxidations were studied by kinetic experiments, gas–liquid chromatography and gas–liquid chromatography–mass spectrometry.

INTRODUCTION

The microbial oxidation of cholesterol to 4-cholesten-3-one was first clearly shown by Turfitt[1] and by Stadtman *et al.*[2]. An extracellular cholesterol oxidase (cholesterol: O_2 oxidoreductase [EC 1.1.3.6]) has recently been demonstrated in cultures of *Streptomyces violascens*[3], and another was isolated and crystallised from the culture fluid of *Brevibacterium sterolicum*[4], the latter enzyme being shown to be a flavoprotein[5]. Cholesterol oxidase isolated from *Nocardia* species has been applied to the clinical estimation of cholesterol[6–8], and is commercially available for this purpose[9–11]. Preliminary studies showed that cholesterol oxidase is not specific for cholesterol[3,6–8] and we have used the enzyme isolated from *Nocardia erythropolis* (Boehringer) in the characterisation of 5-cholestene-3β,26-diol from human brain[12] and in the selective oxidation of other side-chain-hydroxylated 3β-hydroxysteroids[13]. We have also examined the specificity of this enzyme for nuclear analogues of cholesterol and for the effect of side-chain length[14,15], and have exploited differences in the products of the oxidation of 5α- and Δ^5-3β-hydroxysteroids for their resolution by gas–liquid chromatography (GLC)[15]. The slow rate of oxidation of 4-methyl- and C_{19} 3β-hydroxysteroids permits the indirect separation of steroids having similar retention times in GLC[15]. In a further development of this work, we have explored more fully the importance of the C-17 side-chain in the activity of the enzyme, and in its use in the selective oxidation of hydroxysteroids. We have also prepared derivatives

of 17-ketosteroids that are satisfactory substrates for cholesterol oxidase. This has facilitated the oxidation and separation of certain steroids previously shown to be unreactive towards the enzyme[14,15].

MATERIALS

Cholesterol oxidase from *Nocardia erythropolis* (20 U/mg; 1 mg/ml) was kindly supplied by Mr. D. Giles [Boehringer (London), London, Great Britain], and horse-radish peroxidase (H_2O_2 oxidoreductase) [EC 1.11.1.7] (180 U/mg; 1 mg/ml) was obtained from Boehringer Mannheim (Mannheim, G.F.R.).

The hydrochlorides of carboxymethoxyamine, methoxyamine, ethoxyamine, and benzyloxyamine were obtained from Kodak (Kirkby, Great Britain). *sec.*-Butoxyamine and isopentoxyamine hydrochlorides were prepared by Dr. T. A. Baillie. *n*-Butylboronic acid was supplied by Callery (Callery, Pa., U.S.A.). N,O-Bis-(trimethylsilyl)acetamide was obtained from Pierce and Warriner (Chester, Great Britain).

25-Hydroxycholesterol and 24-ketocholesterol were gifts from Dr. G. F. Woods (Organon, Newhouse, Great Britain). Gifts of other steroids were as follows: fucosterol, Dr. P. Bladon; sitosterol, Dr. B. A. Knights; poriferasterol, Dr. L. J. Goad; 19-norstanols and A-norstanols, Prof. L. Minale; fukujusonorone, Prof. H. Mitsuhashi; neoergosterol, Prof. J. D. Loudon (from the collection of Sir James Cook); 15α-hydroxydehydroepiandrosterone and 5-androstene-3β,16β,17β-triol, Dr. E. M. Chambaz; solanidine, solasodine, 19-hydroxy-, 18-hydroxy-, 7α-hydroxy-, 7β-hydroxy-, 11-keto-, and D-homodehydroepiandrosterone and 5-androstene-3β,17β-diol 17-benzoate, M.R.C. Steroid Reference Collection (Prof. W. Klyne and Dr. D. N. Kirk). Other steroids were obtained from BDH (Poole, Great Britain), Farmitalia (Milan, Italy), Koch-Light (Colnbrook, Great Britain), Schwarz/Mann (Orangeburg, N.Y., U.S.A.), Sigma London (London, Great Britain) and Steraloids (Pawling, N.J., U.S.A.). 26-Hydroxycholesterol (m.p. 174–175°) was prepared from kryptogenin according to Scheer *et al.* (m.p. 177–178°)[16]. (22-*RS*)-22-Hydroxycholesterol and (24-*RS*)-24-hydroxycholesterol were prepared from the corresponding side-chain ketones.

METHODS

Incubations

For the determination of kinetic constants, steroids in isopropanol (50 μl) were mixed with 2.75 ml of 50 m*M* NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) containing 1 mg/ml of Triton X-100, 0.1 ml of 4-aminoantipyrine (2.4 m*M*, in buffer) and 0.1 ml of phenol (0.4 *M*, in buffer) in a cuvette of 1 cm light-path maintained at 30°. Incubations were initiated by the addition of horse-radish peroxidase (0.18 U) and cholesterol oxidase (usually 0.02 U) in buffer (10 μl). The rate of hydrogen peroxide production was followed by measuring the increase in extinction at 500 nm, using a Cecil Instruments CE 272 linear readout spectrophotometer linked to a Servoscribe recorder. The initial rates measured for seven substrate concentrations in duplicate, or five in triplicate, were used for the determination of kinetic constants. Provisional values of K_m (μM) and V (μmole min^{-1} mg^{-1} enzyme) were obtained by the graphical

method of Eisenthal and Cornish-Bowden[17] and then estimated more precisely by using a computer program for a least squares adjustment[18] of the Michaelis–Menten curve, using a Digico Micro 16P computer. Relative rates of oxidation of steroids were determined by the addition of steroid (0.26 μmole) in isopropanol (100 μl) to 3 ml of 0.05 *M* phosphate buffer (pH 7.0) containing 0.25 mg/ml of Triton X-100. After the addition of cholesterol oxidase (0.02 U) the rates of steroid Δ^4-3-one production were measured by recording the increases in extinction at 240 nm.

For those incubations that were to be followed by the analysis of products by GLC, steroids (0.13 μmole) in isopropanol (250 μl) were mixed with 3 ml of phosphate buffer and cholesterol oxidase (0.2 U) and the incubations at 30° monitored at 240 nm (when appropriate) to indicate the progress of oxidation.

GLC and GLC–mass spectrometry (MS)

GLC was performed on a Pye 104 gas chromatograph fitted with glass columns of 1.5 m or 3 m length and 4 mm I.D. and packed with 1% OV-1 on Gas-Chrom Q (100–120 mesh) operating at 250, 255, 260 or 275° with nitrogen as the carrier gas (flow-rate 40 or 50 ml/min). Oxime derivatives of dehydroepiandrosterone (Table III) were also chromatographed on a 1.5 m × 4 mm I.D. column of 1% OV-17 on Gas-Chrom Q (100–120 mesh) at 265° with a nitrogen flow-rate of 40 ml/min.

Mass spectra were obtained by GLC–MS using an LKB 9000 instrument equipped with a 1% OV-1 column (3 m) at an electron energy of 70 eV.

Preparation of derivatives

Trimethylsilyl ethers of steroids for GLC were prepared by treating the steroids with N,O-bis(trimethylsilyl)acetamide for 10 min at 60–80°. The oxime or alkyloximes of dehydroepiandrosterone were formed by the reaction of the steroid (25 mg) with hydroxylamine or alkoxyamine hydrochlorides (2–3 molar equivalent) in pyridine (1 ml) overnight at room temperature. After dilution with water, the oximes were extracted with diethyl ether, washed with HCl, NaCl and water. Yields were 80–100% and the oximes were crystallized from methanol–water; m.p. of oxime, 190–192° (ref. 19, 188–191°); methyloxime, 198–200° (ref. 20, 195–196°); ethyloxime, 127–128°; *sec.*-butyloxime, 104–106°; isopentyloxime, 107–109°; benzyloxime, 118–120°. Isopentyloximes for GLC were formed by heating the steroid (50–100 μg) with excess isopentoxyamine hydrochloride in pyridine (100 μl) for 30 min at 60–80°. The *n*-butylboronate of 5-androstene-3β,16β,17β-triol was formed by heating the steroid with *n*-butylboronic acid in pyridine for 30 min at 60°.

RESULTS AND DISCUSSION

Studies of substrate specificity

The enzyme cholesterol oxidase comprises a 3β-hydroxysteroid oxidase and a Δ^5-steroid isomerase component. The oxidation of the 3β-hydroxyl group has been shown to be slower than the isomerisation[21] and in order to measure the rate of oxidation alone, the hydrogen peroxide that was generated was measured by the oxidative coupling of 4-aminoantipyrine with phenol by horse-radish peroxidase, to give a quinone-imine dye absorbing at 500 nm[8,22]. Using this method and under the conditions described in Methods, oxidation was greatest at pH 7.0 and 32°. The addition

TABLE I

APPARENT K_m AND V VALUES FOR THE OXIDATION OF SOME Δ^5-3β-HYDROXYSTEROIDS BY CHOLESTEROL OXIDASE

The assay procedures at 30° and pH 7.0 were as described in Methods.

Δ^5-3β-Hydroxysteroid	$K_m \pm$ S.D. (μM)	$V \pm$ S.D. (μmole min^{-1} mg^{-1})	K_m/V
Cholesterol	2.9 ± 0.2	18.5 ± 0.4	0.16
26-Hydroxycholesterol	1.2 ± 0.05	10.4 ± 0.1	0.12
25-Hydroxycholesterol	1.5 ± 0.2	11.3 ± 0.3	0.13
24-Hydroxycholesterol	4.3 ± 0.3	23.8 ± 0.5	0.18
22-Hydroxycholesterol	5.1 ± 0.4	16.6 ± 0.3	0.31
20-Hydroxycholesterol	4.2 ± 0.3	19.3 ± 0.5	0.22
20,22-Dihydroxycholesterol	7.1 ± 0.5	17.3 ± 0.6	0.41
24-Ketocholesterol	2.7 ± 0.1	24.4 ± 0.2	0.11
Sitosterol	6.6 ± 0.3	19.5 ± 0.5	0.34
Stigmasterol	5.9 ± 0.4	6.4 ± 0.2	0.92
Fucosterol	11.5 ± 1.4	16.3 ± 1.2	0.71
Diosgenin	13.9 ± 0.5	0.60 ± 0.05	23.2
5-Androstene-3β,17β-diol	1.2 ± 0.2	0.11 ± 0.01	10.9
Dehydroepiandrosterone	3.3 ± 0.1	0.17 ± 0.01	19.4
5-Androstene-3β,17β-diol 17-benzoate	5.4 ± 0.5	0.32 ± 0.01	16.9

of NAD^+ caused a slight inhibition, in contrast with other 3-hydroxysteroid oxidoreductases for which it serves as a co-factor[23]. Oxygen has been assumed not to be a rate-limiting factor since at 30° dissolved oxygen should be in substantial excess. Previous studies have demonstrated that changes in the nuclear structure of cholesterol can greatly affect the rate of oxidation and also that the presence of at least a C_2 side-chain at C-17 is required for efficient oxidation[7,14,15]. These differences have been previously exploited in the analysis of steroids by GLC[15]. In the present work various side-chain analogues of cholesterol were examined for their reactivity with cholesterol oxidase by determining K_m and V values (Table I). The position of the side-chain hydroxyl group in hydroxycholesterols influenced the binding and overall rate of oxidation, though the K_m/V values (which may be tentatively regarded as an inverse measure of enzyme efficiency) were very similar. The presence of a hydroxyl group near the terminus of the side-chain (in 26-hydroxycholesterol and 25-hydroxycholesterol) tended to inhibit oxidation, whereas 24-hydroxycholesterol was oxidised 1.3 times as fast as cholesterol. A ketone group at C-24 had a similar enhancing effect, 24-ketocholesterol being the most rapidly oxidised substrate among those studied. The presence of a C-24 ethyl group, as in sitosterol, also appeared to increase the V value, suggesting that a hydrophilic group at C-24 was not obligatory for promoting faster oxidation. However, fucosterol (with a 24-ethylidene group) and stigmasterol (with a C-24 ethyl group and a Δ^{22} bond) were less readily attacked. Diosgenin (with a spiroacetal side-chain) reacted more slowly. 5-Androstene-3β,17β-diol, as previously shown[14], and dehydroepiandrosterone reacted very slowly and gave high K_m/V values.

The oxidations of some other Δ^5-3β-hydroxysteroids were examined by comparing the rates of conjugated enone production (measured at 240 nm) with the rate of formation of 4-cholesten-3-one from cholesterol (Table II). Again sterols with

TABLE II

COMPARATIVE RATES OF OXIDATION OF SOME Δ^5-3β-HYDROXYSTEROIDS

For experimental details see Methods. The absorbances at 240 nm were recorded continuously and the rates of increase in extinction are quoted relative to cholesterol (3–8 independent incubations).

Steroid	*Relative rate*
Cholesterol	100
22-Ketocholesterol	93
Kryptogenin	92
Poriferasterol	68
Solasodine	<0.1
Solanidine	<0.1
3β-Hydroxycholenoic acid	22
Pregnenolone	82
1β-Hydroxypregnenolone	14
5-Pregnene-3β,20α-diol	24
5-Pregnene-3β,20β-diol	27
5-Pregnen-3β-ol	6
5-Pregnene-3β,17α,20α-triol	5
Dehydroepiandrosterone carboxymethyloxime	0.1

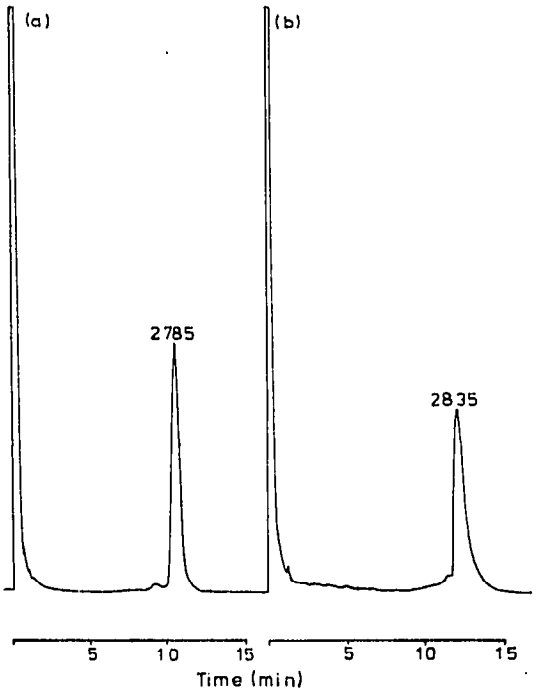

Fig. 1. GLC of 5,16-pregnadiene-3β,20α-diol (a) before incubation with cholesterol oxidase; (b) after incubation. Steroids were chromatographed as their trimethylsilyl ethers. Column: 3 m × 4 mm I.D., 1% OV-1 operating at 250° with 50 ml/min of nitrogen.

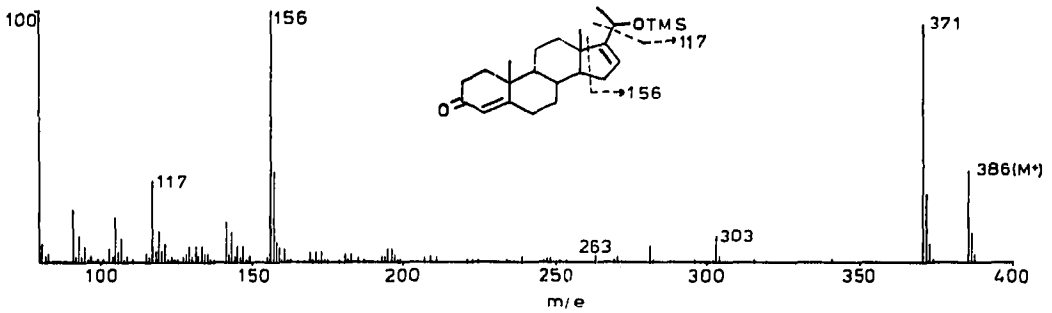

Fig. 2. Mass spectrum of 20α-hydroxy-4,16-pregnadien-3-one (as the trimethylsilyl ether), formed by oxidation of 5,16-pregnadiene-3β,20α-diol by cholesterol oxidase.

side-chains similar to cholesterol were oxidised at comparable rates. The alkaloids solasodine (structurally analogous to diosgenin) and solanidine were virtually unattacked by the enzyme. The pregnenes examined were all oxidised more slowly than cholesterol, as expected from the short side-chain length, though pregnenolone was a much better substrate than the 20-hydroxy analogues. The presence of a 1β-hydroxyl group (in 1β-hydroxypregnenolone) hindered oxidation.

The oxidations of other potential substrates were examined by GLC. Only partial oxidation of neoergosterol and of fukujusonorone (3β-hydroxy-18-nor-5,13-pregnadiene-12,20-dione)[24] occurred overnight, and as expected, oestrone, having an aromatic ring A and no 19-methyl group or side-chain, did not serve as a substrate. A mixture of C_{26}, C_{27}, C_{28} and C_{29} 19-norstanols isolated from the sponge *Axinella polypoides*[25] was oxidised at a rate 30% that of cholesterol (as determined by the peroxidase coupling method). GLC showed that complete oxidation had occurred. In contrast, a mixture of C_{26}, C_{27}, C_{28} and C_{29} A-norstanols from the sponge *Axinella verrucosa*[26] remained unchanged after incubation with cholesterol oxidase.

As an example of the use of cholesterol oxidase in the selective oxidation of hydroxysteroids, 5,16-pregnadiene-3β,20α-diol was incubated with the enzyme. Thin-layer chromatography followed by detection with 2,4-dinitrophenylhydrazine and GLC of the trimethylsilyl ether (Fig. 1) showed only one product, corresponding to

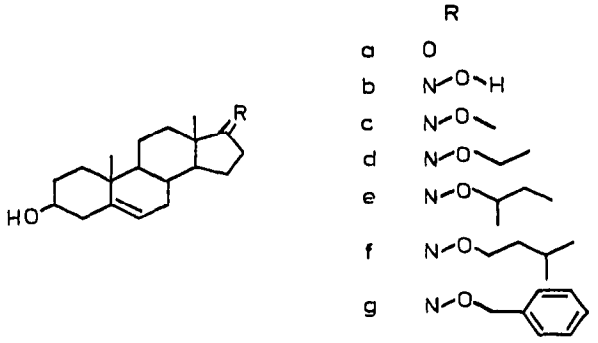

Fig. 3. Structural formulas of (a) dehydroepiandrosterone; (b) oxime; (c) methyloxime; (d) ethyloxime; (e) *sec.*-butyloxime; (f) isopentyloxime; (g) benzyloxime.

TABLE III

GLC AND GLC–MS DATA FOR OXIMES OF DEHYDROEPIANDROSTERONE (DHA) AND THEIR TRIMETHYLSILYL ETHERS

Column lengths, 1.5 m.

Substrate	$I^{255°}_{OV\text{-}1}$	$I^{265°}_{OV\text{-}17}$	*Trimethylsilyl ethers*							
			$I^{255°}_{OV\text{-}1}$	$I^{265°}_{OV\text{-}17}$	*Mol. wt.*	*Base peak above m/e 80*	*Relative abundance of ions*			
							$M^{+\cdot}$	$M - 129$	*m/e 358***	*m/e 268****
DHA	2550	2940	2600	2850	360	129	6	12		
Oxime	(2595)*	3000	(3735)*	2890	447	129	6	16	23	36
Methyloxime	2590	2950	2635	2870	389	129	5	25	15	20
Ethyloxime	2530	2980	2695	2910	405	129	3	10	28	48
sec.-Butyloxime	2760	3095	2810	3010	431	129	7	12	32	66
Isopentyloxime	2905	3245	2955	3165	445	129	3	10	7	25
Benzyloxime	3260	3730	3305	3645	465	91	1	9	21	24

* Data not accurate owing to decomposition during GLC.
** $[M^{+\cdot} - RO\cdot]$: cleavage at oxime group.
*** $[M^{+\cdot} - RO\cdot - (CH_3)_3SiOH]$.

20α-hydroxy-4,16-pregnadien-3-one. No dienedione was detected. Confirmation of the structure of the product was obtained by GLC–MS (Fig. 2).

Studies of isopentyloximes and related derivatives

It appeared possible that the rates of oxidation of 5-androsten-17-ones might be improved by formation of alkyloximes, yielding side-chains simulating those of sterols. On this assumption, it was presumed that the isopentyloxime (Fig. 3f) would be the most effective substrate. In a comparative study, the oxime, methyloxime, ethyloxime, *sec.*-butyloxime, isopentyloxime and benzyloxime of dehydroepiandrosterone (Fig. 3a–g) were prepared and characterised by GLC and GLC–MS (Table III). The base peak (above *m/e* 80) of the mass spectra was *m/e* 129 except for the benzyloxime, where the benzyl moiety (*m/e* 91) gave rise to the base peak. Using the

TABLE IV

APPARENT K_m AND V VALUES FOR THE OXIDATION OF DEHYDROEPIANDROSTERONE OXIME AND ALKYL OXIMES BY CHOLESTEROL OXIDASE

The assay procedure at 30° and pH 7.0 was as described in Methods.

Substrate	$K_m \pm$ *S.D.* *(μM)*	$V \pm$ *S.D.* *(μmole min^{-1} mg^{-1})*	K_m/V
DHA	3.3 ± 0.1	0.17 ± 0.01	19.4
Oxime	4.9 ± 0.8	0.09 ± 0.01	54.4
Methyloxime	1.8 ± 0.1	0.15 ± 0.01	12.0
Ethyloxime	1.9 ± 0.2	0.27 ± 0.01	7.0
sec.-Butyloxime	23.8 ± 2.3	0.71 ± 0.04	33.5
Isopentyloxime	27.1 ± 1.8	5.7 ± 0.2	4.6
Benzyloxime	22.5 ± 1.5	11.0 ± 0.4	2.0

peroxidase coupling method, the kinetic constants of the oximes were determined with cholesterol oxidase (Table IV). Only the isopentyloxime and benzyloxime served to mimic the cholesterol side-chain by giving satisfactory rates of oxidation, though in both cases approximately a ten-fold increase in the K_m value relative to cholesterol was observed. Though the benzyloxime appeared, in fact, to be the better substrate, the isopentyloxime was chosen for further investigations because the benzyloxime gives much longer retention times on GLC (Table III); moreover, the isopentyloximes have been of interest in our laboratory in connection with urinary steroid analysis[27,28]. The following examples illustrate potential applications of these derivatives.

The trimethylsilyl ethers of dehydroepiandrosterone and epiandrosterone are poorly separated on packed columns of OV-1 and OV-17. Separation in the form of the corresponding Δ^4- and 5α-3-ketones is precluded by the low reactivity of the free steroids with cholesterol oxidase. The derived isopentyloximes are oxidised by the enzyme much faster than the parent steroids. The trimethylsilyl ethers of the resulting 17-isopentyloximes of 5α-androstane-3,17-dione and 4-androstene-3,17-dione are well separated by GLC (Fig. 4) in accord with previous studies[15].

The isopentyloximes of some analogues of dehydroepiandrosterone were also treated with cholesterol oxidase. The retention indices of trimethylsilyl ethers on GLC before and after oxidation were measured to demonstrate that oxidation had taken place (Table V). The products were also identified by GLC–MS. The 3β-hydroxy

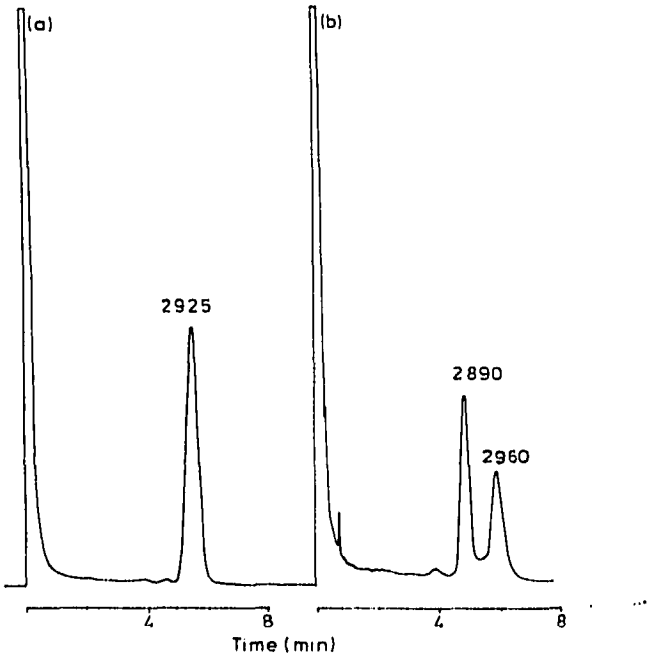

Fig. 4. GLC separation of isopentyloxime derivatives of dehydroepiandrosterone and 3β-hydroxy-5α-androstan-17-one (a) as trimethylsilyl ethers; (b) after incubation with cholesterol oxidase. Column: 1.5 m × 4 mm I.D., 1% OV-1 at 260° with 40 ml/min of nitrogen.

TABLE V

RETENTION INDICES FOR ANALOGUES OF DEHYDROEPIANDROSTERONE AS ISOPENTYLOXIMES, BEFORE AND AFTER OXIDATION WITH CHOLESTEROL OXIDASE

For experimental details see Methods. Hydroxysteroids were chromatographed as trimethylsilyl ethers.

Substrate	$I^{275°}_{OV\text{-}1}$	
	Before oxidation	*After oxidation*
DHA isopentyloxime	2980	3045
19-Hydroxy-DHA isopentyloxime	3055	3200
18-Hydroxy-DHA isopentyloxime	3105	3165
15α-Hydroxy-DHA isopentyloxime	2970	3070
7α-Hydroxy-DHA isopentyloxime	2980	3070
7β-Hydroxy-DHA isopentyloxime	3090	3140
11-Keto-DHA isopentyloxime	3070	3115
7-Keto-DHA diisopentyloxime	3400	3400*
D-Homo-DHA isopentyloxime	3085	3165

* Unchanged substrate.

group was selectively oxidised in the presence of other hydroxyl groups (7α-, 7β-, 15α-, 18- and 19-) which would have been susceptible to oxidation by chemical methods. The example of 18-hydroxydehydroepiandrosterone is illustrated in Fig. 5. 7-Keto-dehydroepiandrosterone formed a diisopentyloxime which was unattacked by the

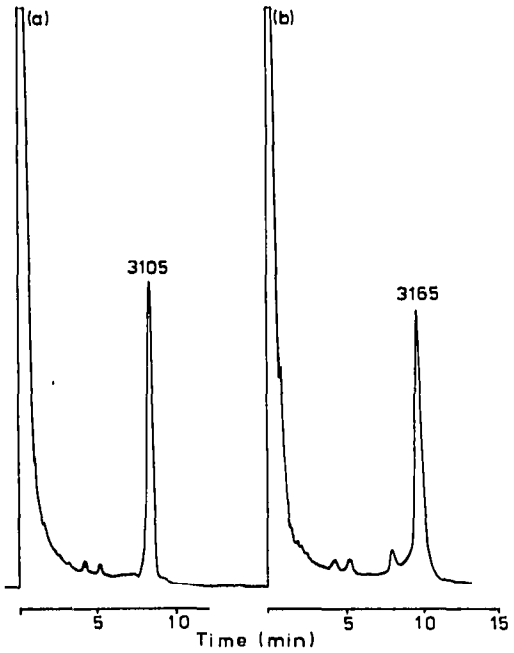

Fig. 5. GLC of the trimethylsilyl ethers of (a) 18-hydroxydehydroepiandrosterone; (b) product after incubation with cholesterol oxidase. GLC conditions as in Table V.

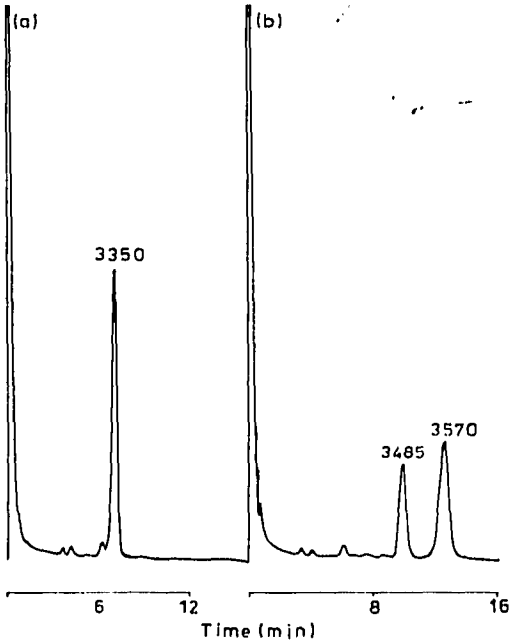

Fig. 6. GLC separation of the isopentyloximes of pregnenolone and 3β-hydroxy-5α-pregnan-3-one (a) as trimethylsilyl ethers; (b) after incubation with cholesterol oxidase. Column: 1.5 m × 4 mm I.D., 1% OV-17 at 260° and 40 ml/min of nitrogen.

enzyme (Table V): inhibition of oxidation by modification of nuclear functional groups thus affords another possible basis of separation.

Urinary steroid mixtures contain Δ^5- and 5α-20-ketones of the pregnane series, which are relatively efficiently oxidised by the enzyme (Table II). However, the product of isopentyloximation of urinary steroid mixtures would include 20-isopentyloximes. Accordingly, pregnenolone isopentyloxime and 3β-hydroxy-5α-pregnan-20-one isopentyloxime were tested as substrates for cholesterol oxidase. Though the steroids were not satisfactorily resolved during GLC as their trimethylsilyl ethers, complete separation was achieved after incubation with the enzyme (Fig. 6).

In applications of isopentyloxime trimethylsilyl ethers to urinary steroid analysis, fortuitous coincidence of peaks from ketonic and non-ketonic steroids may occur. Enzymic oxidation can sometimes resolve this difficulty. Thus 5-pregnene-3β,17α,20β-triol and 11-ketoandrosterone (as trimethylsilyl ethers) are not resolved during GLC, but prior incubation with the enzyme led to a separation (Fig. 7): 11-ketoandrosterone, as a 3α-hydroxysteroid, was unaffected[14].

Other derivatives were briefly examined. The carboxymethyloxime of dehydroepiandrosterone was a very poor substrate (Table II). 5-Androstene-3β,17β-diol 17-benzoate was oxidised only slightly faster than the free diol. The 16β,17β-*n*-butylboronate of 5-androstene-3β,16β,17β-triol was ineffective in simulating the sterol side-chain, the derivative being oxidised only twice as fast as the parent triol.

CONCLUSIONS

Current interest in cholesterol oxidase centres on its use in the clinical estima-

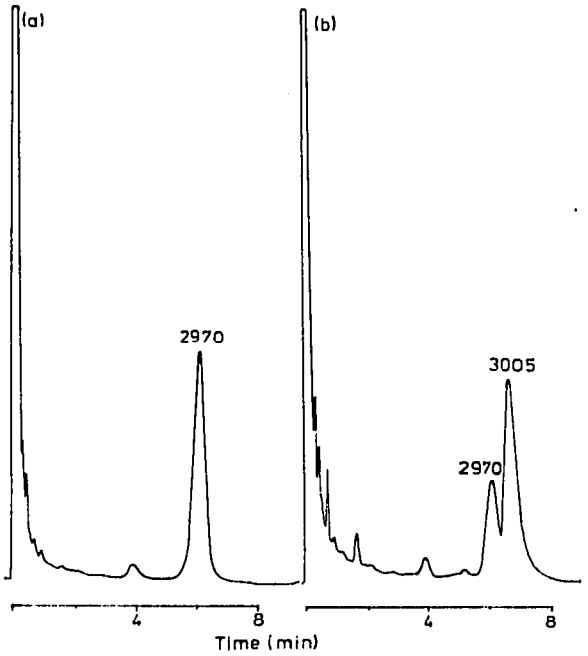

Fig. 7. GLC of the trimethylsilyl ethers of (a) 5-pregnene-3β,17α,20β-triol and the 17-isopentyloxime of 3α-hydroxy-5α-androstane-11,17-dione; (b) the products after incubation with cholesterol oxidase. GLC conditions as in Fig. 4.

tion of cholesterol. However, we have demonstrated the potentially wide applications of the enzyme in the analysis of other Δ^5- and 5α-3β-hydroxysteroids[15]. The data presented above provide extended knowledge of the substrate specificity of the enzyme (especially in respect of side-chain requirements). In addition, it is shown that by using isopentyloxime derivatives of 17-ketosteroids, relatively unreactive Δ^5- or 5α-3β-hydroxysteroids can be smoothly oxidised to Δ^4- and 5α- 3-ketones, respectively, without concomitant alteration of other hydroxyl groups. The methods that have been developed are applicable to the study of complex sterol mixtures (such as those of marine organisms) and to investigations of urinary steroids, particularly those of newborn infants in which Δ^5- and 5α-3β-hydroxysteroids occur in substantial proportions. The specificity of cholesterol oxidase, in respect of natural and suitably modified substrates, provides a simple and powerful method of selective oxidation under extremely mild conditions. The oxidative elaboration of the Δ^4-3-keto group in the presence of hydroxylic substituents elsewhere in the steroid molecule is of special interest, in view of the absence of a chemical oxidant of comparable selectivity and efficiency.

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SYSTEMATIC NAMES OF STEROIDS

Cholesterol = 5-cholesten-3β-ol
Dehydroepiandrosterone = 3β-hydroxy-5-androsten-17-one
20,22-Dihydroxycholesterol = (20*R*,22*R*)-5-cholestene-3β,20,22-triol
Diosgenin = (25*R*)-5-spirosten-3β-ol
Epiandrosterone = 3β-hydroxy-5α-androstan-17-one
Fucosterol = 5,*E*-24(28)-stigmastadien-3β-ol
D-Homodehydroepiandrosterone = 3β-hydroxy-D-homo-5-androsten-17a-one
3β-Hydroxycholenoic acid = 3β-hydroxy-5-cholen-24-oic acid
20-Hydroxycholesterol = (20*S*)-5-cholestene-3β,20-diol
22-Hydroxycholesterol = 5-cholestene-3β,22-diol
24-Hydroxycholesterol = 5-cholestene-3β,24-diol
26-Hydroxycholesterol = 5-cholestene-3β,26-diol
7α-Hydroxydehydroepiandrosterone = 3β,7α-dihydroxy-5-androsten-17-one
7β-Hydroxydehydroepiandrosterone = 3β,7β-dihydroxy-5-androsten-17-one
15α-Hydroxydehydroepiandrosterone = 3β,15α-dihydroxy-5-androsten-17-one
18-Hydroxydehydroepiandrosterone = 3β,18-dihydroxy-5-androsten-17-one
19-Hydroxydehydroepiandrosterone = 3β,19-dihydroxy-5-androsten-17-one
1β-Hydroxypregnenolone = 1β,3β-dihydroxy-5-pregnen-20-one
11-Ketoandrosterone = 3α-hydroxy-5α-androstane-11,17-dione
22-Ketocholesterol = 3β-hydroxy-5-cholesten-22-one
24-Ketocholesterol = 3β-hydroxy-5-cholesten-24-one
Kryptogenin = (25*R*)-3β,26-dihydroxy-5-cholesten-16,22-dione
Neoergosterol = (24*R*)-19-nor-5,7,9(10),22-ergostatetraen-3β-ol
Oestrone = 3-hydroxy-1,3,5-estratrien-17-one
Poriferasterol = (24*R*)-5,22-stigmastadien-3β-ol
Pregnenolone = 3β-hydroxy-5-pregnen-20-one
Sitosterol = (24*R*)-5-stigmasten-3β-ol
Solanidine = (22*S*,25*S*)-5-solanidenin-3β-ol
Solasodine = (22*S*,25*S*)-5-tomatenin-3β-ol
Stigmasterol = (24*S*)-5,22-stigmastadien-3β-ol

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