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APPLICATION OF CHOLESTEROL OXIDASE IN THE ANALYSIS OF STEROIDS

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SUMMARY

Cholesterol oxidase has been used in the qualitative analysis of model mixtures of hydroxylic steroids. Selective oxidation of Δ^5-3β-ols and 5α-3β-ols of the sterol series has been accomplished: the resulting ketones were completely separable by gas–liquid chromatography. Steroids with the cholane and pregnane types of side-chain were oxidised more slowly than cholesterol and those of the androstane group were almost unattacked. Steroids with modified sterol side-chains (C-24 alkylated sterols, hydroxycholesterols, sapogenins) were satisfactory substrates.

INTRODUCTION

Microbial enzymes capable of oxidising cholesterol have been known for many years[1], but enzyme preparations that convert cholesterol to cholest-4-en-3-one in high yield have only recently become readily available[2–4]. These have been developed primarily for the determination of cholesterol in blood serum[5–7]. Methods of measurement may be based either on the absorbance of cholest-4-en-3-one at 240 nm, or on estimation of the hydrogen peroxide formed during the enzymic oxidation.

Preliminary reports[5–7] on cholesterol oxidase derived from *Nocardia* species have indicated that the enzyme is not specific for cholesterol. In particular, certain other Δ^5-3-hydroxysteroids and 5α-cholestan-3β-ol were susceptible to oxidation. These observations prompted us to examine the potential of the enzyme for the indirect analysis of certain mixtures of sterols that are not well separated under commonly used gas–liquid chromatographic (GLC) conditions. Sterols of the 3β-hydroxy-Δ^5- and 3β-hydroxy-5α- types, for example, co-occur in sterol mixtures from many natural sources, and their satisfactory separation by GLC demands the use of columns of very high resolving power. Selective enzymic oxidation, as outlined below, provides a simple and convenient means of effecting certain separations, and of enhancing the information obtainable by GLC–mass spectrometry (MS). The latter aspect has been exemplified in our recent characterisation of 26-hydroxycholesterol isolated from human brain[8].

EXPERIMENTAL

Materials

Cholesterol oxidase (EC 1.1.3.6) from *Nocardia erythropolis* was kindly supplied by Mr. D. Giles [Boehringer Corporation (London), London, Great Britain] as a solution in 0.1 *M* phosphate buffer (pH 5.0) containing 1% *tert.*-butan-1-ol (for stabilisation). This preparation contained 1 mg of protein per ml, and had a stated activity of 20 I.U. per mg of protein when assayed at 37°. Contaminating enzymes stated to be present at concentrations less than 0.01% were glucose oxidase (EC 1.1.3.4), peroxidase (EC 1.11.1.7) and catalase (EC 1.11.1.6).

Ergostanol, campesterol, α-spinasterol, sitosterol and stigmastanol were gifts from Dr. B. A. Knights; lophenol, from the M.R.C. Steroid Reference Collection (Prof. W. Klyne and Dr. D. N. Kirk); desmosterol and 24-oxocholesterol, from Dr. G. F. Woods (Organon, Newhouse, Great Britain). 25-Hydroxy-27-norcholesterol, chol-5-ene-3β,24-diol, 23,24-dinorchol-5-ene-3β,22-diol and 17β-(hydroxymethyl)-androst-5-en-3β-ol were prepared by hydride reduction ($LiAlH_4$) of the corresponding side-chain ketones or bile acid methyl esters.

Methods

Spectrophotometric measurements were made with reference to "blanks" containing all components except the substrate, using a Cecil Instruments CE 272 Linear Readout Spectrophotometer. GLC was carried out using a Pye 104 instrument with a flame ionisation detector. The silanized glass column (3 m × 3 mm I.D.) was packed with 1% OV-1 on Gas-Chrom Q (100–120 mesh) (Applied Science Labs., State College, Pa., U.S.A.). Incubations to determine the relative rates of oxidation of Δ^5-3β-ols were as follows: Steroids (0.26 μmole) were added in isopropanol (100 μl) to 3 ml of 0.05 *M* phosphate buffer (pH 7.0) containing Triton X-100 (3 mg), in a cuvette (1-cm light path). Cholesterol oxidase freshly diluted with 0.05 *M* phosphate buffer (1:10; 10 μl) was then added and the mixture incubated at 37°. The extinction at 240 nm was monitored continuously and the maximum rate of change of extinction was measured. For incubations to be followed by GLC, steroids (0.13 μmole) were added in isopropanol (250 μl) to 0.05 *M* phosphate buffer (pH 7.0; 3 ml). Cholesterol oxidase (10 μl) was then added, and the mixture incubated at 37° for 1½ h. Continuous monitoring by UV at 240 nm enabled recognition of the end-point of any Δ^5-3β-ol oxidation. The steroids were then extracted with diethyl ether (2 ml), the extracts were evaporated to dryness and the residues dissolved in ethyl acetate (50 μl). Aliquots (1 or 2 μl) were taken for GLC and GLC–MS. GLC–MS was carried out using an LKB 9000 instrument at 70 eV. The column packing and conditions were the same as those used for GLC.

RESULTS

Initial studies with the enzyme demonstrated that optimal oxidation of cholesterol in Triton X-100 and isopropanol was observed at pH 7.0, and an apparent K_m of approximately 7 μ*M* was determined under these conditions. 3β-Hydroxy-5α-sterols were oxidised, though at a lower rate than their Δ^5-analogues. To determine the relative rates of oxidation of cholesterol and cholestanol, these were incubated

simultaneously and the oxidation of cholesterol was monitored by the increase in UV extinction at 240 nm. When the rate of formation of cholestenone ceased to be linear, the incubations were terminated and the relative degrees of oxidation of the two sterols determined by GLC. The extent of oxidation of cholestanol was approximately 85% of that of cholesterol.

Sterols and steroids with a variety of side-chains were observed to be oxidised by the enzyme, and the influence of side-chain length on the rate of oxidation of Δ^5-3β-hydroxy steroids was investigated. Seven diols containing the Δ^5-3β-hydroxy grouping were treated with the enzyme, and the rate of Δ^4-3-one formation, as monitored by the increase in extinction at 240 nm, was compared with that of cholesterol (Table I). We have observed that a hydroxy group in the 25- or 26-position of the cholesterol side-chain decreased the affinity of the enzyme for a steroid, as illustrated here by the difference in rates between cholesterol and 26-hydroxycholesterol. Though the series in Table I is not completely homologous, it shows that at least a C_2 side-chain is required for a satisfactory rate of oxidation of the 3β-hydroxy group in these steroid diols. 17β-(Hydroxymethyl)-androst-5-en-3β-ol and androst-5-ene-3β,17β-diol were relatively little attacked by the enzyme (Table I) and this feature can be used in separating these compounds from those more susceptible to the enzyme (Table II). Cholest-4-en-3β-ol was oxidised at a rate comparable with that for cholesterol. In contrast, the presence of 4,4-dimethyl substituents almost completely inhibited 3β-hydroxy oxidation. In order to determine the susceptibility of a 3α-hydroxy group to oxidation, epicholesterol and epicholestanol were treated with the enzyme. The rates of oxidation were found to be less than 1% of those of the 3β-epimers.

5α-Cholestanol and cholesterol are not satisfactorily separable on many of the phases commonly used for GLC. However, the products of oxidation, 5α-cholestan-3-one and cholest-4-en-3-one, show markedly different behaviour both in GLC and thin-layer chromatography. The enzymic oxidation can be applied to the separation of many other pairs of steroids. An incubation time of 1½ h was found to be convenient for the oxidation of 100-μg amounts of steroids, though oxidation of C_{27}, C_{28} and C_{29} sterols was usually completed more quickly.

TABLE I

COMPARATIVE RATES OF OXIDATION OF 3β-HYDROXY-Δ^5-STEROIDS

For experimental details, see *Methods*. The extinctions at 240 nm were recorded continuously and the rates of increase in extinction are quoted relative to cholesterol (mean values of 5–8 independent incubations).

Steroid	*No. of carbon atoms in side-chain*	*Relative rate*
Cholesterol	8	100
26-Hydroxycholesterol	8	67
25-Hydroxy-27-norcholesterol	7	69
Chol-5-ene-3β,24-diol	5	71
23,24-Dinorchol-5-ene-3β,22-diol	3	35
Pregn-5-ene-3β,20α-diol	2	24
17β-(Hydroxymethyl)-androst-5-en-3β-ol	1	1
Androst-5-ene-3β,17β-diol	0	1

TABLE II

RETENTION INDICES FOR PAIRS OF STEROIDS AND FOR THE PRODUCTS ISOLATED AFTER INCUBATION WITH CHOLESTEROL OXIDASE

For experimental details, see *Methods*.

*No.**	*Starting material*	*Retention index*	*Product*	*Retention index*
(A) Sterols (column temperature, 275°)				
1	Cholesterol	3105	Cholestenone	3220
2	Cholestanol	3115	Cholestanone	3145
3	7-Dehydrocholesterol	3135	Cholesta-4,7-dien-3-one (?)**	3300
4	Cholest-7-en-3β-ol	3135	Cholest-7-en-3-one	3165
3	7-Dehydrocholesterol	3130	Cholesta-4,7-dien-3-one (?)**	3285
5	Desmosterol	3125	Cholesta-4,24-dien-3-one	3230
6	24-Oxocholesterol	3300	24-Oxocholestenone	3425
7	Stigmastanol	3290	Stigmastanone	3315
8	Campesterol	3210	Campestenone	3320
9	Ergostanol	3210	Ergostanone	3240
8	Campesterol	3215	Campestenone	3325
10	Ergost-8(14)-en-3β-ol	3215	Ergost-8(14)-en-3-one	3230
8	Campesterol	3215	Campestenone	3320
11	Lophenol	3215	Lophenone	3210
			Lophenol	3215
12	Sitosterol	3290	Sitostenone	3405
7	Stigmastanol	3300	Stigmastanone	3325
13	Fucosterol	3280	Fucosterone	3390
7	Stigmastanol	3290	Stigmastanone	3315
12	Sitosterol	3285	Sitostenone	3395
14	α-Spinasterol	3270	α-Spinasterone	3295
15	Stigmasterol	3240	Stigmasterone	3355
16	4,4-Dimethylcholesterol	3255	(Unchanged)	3255
17	Diosgenin	3255	Diosgenone	3360
18	Tigogenin	3260	Tigogenone	3295
(B) Steroids converted to trimethylsilyl ethers for GLC (column temperature, 250°)				
19	Androst-5-ene-3β,16α-17α-triol	2825	(Unchanged)	2830
20	Pregn-5-ene-3β,20β-diol	2825	20β-Hydroxypregn-4-en-3-one	2870
21	Pregn-5-ene-3β,20α-diol	2860	20α-Hydroxypregn-4-en-3-one	2910
22	*allo*-Pregnane-3β,20α-diol	2865	20α-Hydroxy-5α-pregnan-3-one	2835
20	Pregn-5-ene-3β,20β-diol	2835	20β-Hydroxypregn-4-en-3-one	2875
23	*allo*-Pregnane-3β,20β-diol	2840	20β-Hydroxy-5α-pregnan-3-one	2805
24	Pregnenolone	2740	Progesterone	2795
25	*allo*-Pregnanolone	2740	5α-Pregnane-3,20-dione	2720
26	17α-Hydroxypregnenolone	2800	17α-Hydroxyprogesterone	2885
27	17α-Hydroxy*allo*-pregnanolone	2800	17α-Hydroxy-5α-pregnane-3,20-dione	2825

* Systematic names of steroids: 1 = Cholest-5-en-3β-ol; 2 = 5α-cholestan-3β-ol; 3 = cholesta-5,7-dien-3β-ol; 4 = 5α-cholest-7-en-3β-ol; 5 = cholesta-5,24-dien-3β-ol; 6 = 3β-hydroxycholest-5-en-24-one; 7 = (24R)-24-ethyl-5α-cholestan-3β-ol; 8 = (24R)-24-methylcholest-5-en-3β-ol; 9 = (24S)-24-methyl-5α-cholestan-3β-ol; 10 = (24S)-24-methyl-5α-cholest-8(14)-en-3β-ol; 11 = 4α-methyl-5α-cholest-7-en-3β-ol; 12 = (24R)-24-ethylcholest-5-en-3β-ol; 13 = E-24-ethylidenecholest-5-en-3β-ol; 14 = (24S)-24-ethylcholesta-7,22-dien-3β-ol; 15 = (24S)-24-ethylcholesta-5,22-dien-3β-ol; 16 = 4,4-dimethylcholest-5-en-3β-ol; 17 = (25R)-spirost-5-en-3β-ol; 18 = (25R)-5α-spirostan-3β-ol; 22 = 5α-pregnane-3β,20α-diol; 23 = 5α-pregnane-3β,20β-diol; 24 = 3β-hydroxypregn-5-en-20-one; 25 = 3β-hydroxy-5α-pregnan-20-one; 26 = 3β,17α-dihydroxypregn-5-en-20-one; 27 = 3β,17α-dihydroxy-5α-pregnan-20-one.

** These products have not been characterised by GLC–MS.

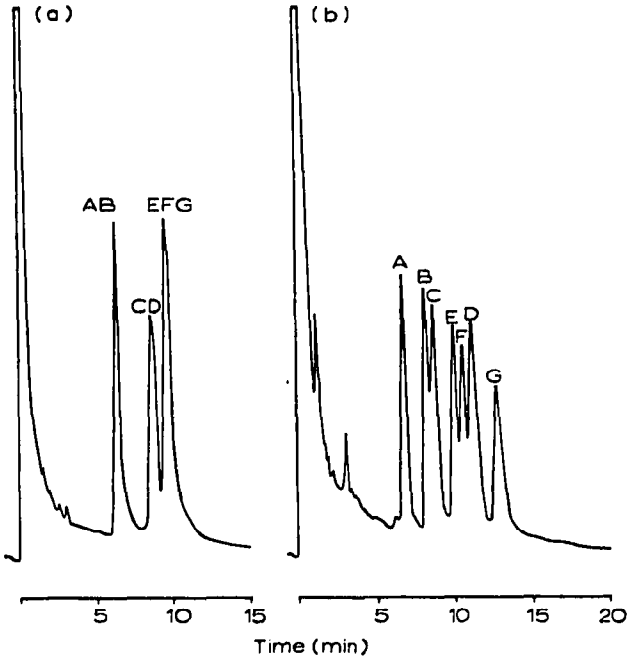

Fig. 1. GLC separation of seven sterols: (a) before incubation with cholesterol oxidase; (b) after incubation. Column: 3 m × 3 mm I.D. 1% OV-1 on Gas-Chrom Q, operating at 275° with an argon flow-rate of 50 ml/min. Incubation conditions were as described in *Methods*. Chromatogram a: A = Cholestanol; B = cholesterol; C = 4,4-dimethylcholesterol; D = stigmasterol; E = α-spinasterol; F = stigmastanol; G = fucosterol. Chromatogram b: Compounds are the oxidation products of the sterols as given in Table II.

Illustrative analytical separations based on GLC are given in Table II. The steroids in Section A were analysed directly, and those in Section B after trimethylsilylation. Most of the examples are based upon the production of 5α-3-ketones and Δ^4-3-ketones. However, the distinction between stigmasterol and 4,4-dimethylcholesterol depends on the unreactivity of the 4,4-substituted sterol. Similarly, androst-5-ene-3β,16α,17α-triol separates from pregn-5-ene-3β,20β-diol after incubation because of the unreactivity of the former steroid. The use of the enzyme to separate more complicated mixtures is illustrated in Fig. 1. Seven sterols (cholestanol, cholesterol, 4,4-dimethylcholesterol, stigmasterol, α-spinasterol, stigmastanol and fucosterol) gave only three peaks during GLC on 1% OV-1. After treatment with cholesterol oxidase, seven peaks were observed. In incubations involving GLC, the identities of the products were confirmed by comparison of retention times with standards, and by GLC–MS.

DISCUSSION

In order to assess the analytical value of cholesterol oxidase, a firm knowledge of substrate specificity is needed. Our studies indicate that the length of the steroid side-chain is a dominant factor affecting the rate of oxidation of a 3β-hydroxy group. The position and type of side-chain oxygenation also have profound effects, which

are being further investigated. The general lack of reactivity of androstenes towards the enzyme affords a possible means of separating these from higher steroids (*e.g.* in urinary steroid extracts). We envisage that cholesterol oxidase will be immediately useful for the analysis of complex mixtures of sterols —for example, sterols of marine organisms, in which small proportions of 3β-hydroxy-5α-sterols are often found in admixture with the corresponding 3β-hydroxy-Δ^5-sterols.

The enzyme may also be convenient for the 'screening' of plant material for important 3β-hydroxy-Δ^5-sterols such as diosgenin. This steroid was found to be completely oxidised under the standard conditions, affording diosgenone as the major product. More complex results have been reported for other microbial oxidations of diosgenin[9,10].

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