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The Substrate Specificity and Stereochemistry, Reversibility and Inhibition of the 3-Oxo Steroid Δ^4–Δ^5-Isomerase Component of Cholesterol Oxidase

By ANDREW G. SMITH and CHARLES J. W. BROOKS
Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

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1. 5-Cholesten-3-one was shown to be an intermediate in the conversion of cholesterol into 4-cholesten-3-one by *Nocardia* cholesterol oxidase. 2. The absence of a C-17 side chain from 5-androstene-3,17-dione slightly increased the $V_{max.}$ of the isomerase activity relative to 5-cholesten-3-one (1.7-fold), but greatly increased the K_m. 3. Incubations of [4α-^{2}H]- and [4β-^{2}H]-cholesterol with cholesterol oxidase showed that the 4β-hydrogen atom can be transferred to the 6β-position. However, incubations of cholesterol, 5-cholesten-3-one and 4-cholesten-3-one with the enzyme in ^2H_2O led to some incorporation of ^{2}H into the 4-cholesten-3-one products, mostly at position 6β. 4. Both the isomerase and the oxidase activities of cholesterol oxidase were inhibited by 5,10-seco-19-nor-5-cholestyne-3,10-dione.

The use of cholesterol oxidase (cholesterol–O_2 oxidoreductase, EC 1.1.3.6) for the determination of serum cholesterol is now widespread (for reviews see Smith & Brooks, 1976*a*, 1977*a*). Three seemingly different types of the enzyme have been reported, namely those from *Nocardia* spp. (Flegg, 1973; Richmond, 1973), *Brevibacterium sterolicum* (Uwajima *et al.*, 1973) and *Streptomyces* spp. (Fukuda *et al.*, 1973; Kerényi *et al.*, 1975; Tomioka *et al.*, 1976). The enzyme converts cholesterol into 4-cholesten-3-one with concomitant production of H_2O_2. The process can be envisaged as oxidation of the 3β-hydroxy group followed by isomerization of the Δ^5-bond. 5-Cholesten-3-one is therefore implicated as an intermediate in this sequence. The substrate specificity of the cholesterol oxidase from *Nocardia erythropolis* for 3β-hydroxy steroid oxidation has been studied in some detail (Brooks & Smith, 1975; Smith & Brooks, 1975, 1976*a*; Wortberg, 1975; Peynet *et al.*, 1976). The length and type of the side chain at position C-17 of a 3β-hydroxy steroid were found to influence considerably the rate of oxidation. We have now demonstrated the intermediacy of 5-cholesten-3-one in the conversion of cholesterol into 4-cholesten-3-one by the cholesterol oxidase from *N. erythropolis*, and have explored the effect of C-17 side-chain length on the rate of isomerization of 3-oxo Δ^5-steroids. We have also demonstrated some stereoselective features of the isomerization, and have studied the inhibition of the enzyme by acetylenic seco-steroids (Batzold & Robinson, 1975, 1976).

Experimental

Steroids

Cholesterol, 4-cholesten-3-one, 5-cholesten-3-one, 3β-hydroxy-5-pregnen-20-one (pregnenolone), 4-pregnene-3,20-dione (progesterone), 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone) and 4-androstene-3,17-dione were from BDH Chemicals (Poole, Dorset, U.K.). 5-Androstene-3,17-dione was a gift from Dr. P. Talalay, and 3β-hydroxy-5,10-seco-19-nor-5-cholestyn-10-one and 5,10-seco-19-nor-5-cholestyne-3,10-dione were gifts from Dr. C. H. Robinson (Batzold & Robinson, 1976). [Both donors are at the Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.] [4-^{14}C]Cholesterol (61 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. All steroids were purified by t.l.c. before use and recrystallized where appropriate.

Preparation of 4-[4-^{14}*C*]*cholesten-3-one*

[4-^{14}C]Cholesterol (10μCi) in propan-2-ol (50μl) was mixed with 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0 (3ml), and incubated with cholesterol oxidase (10μl) for 3h at 25°C. 4-Cholesten-3-one (100μg) and 5-cholesten-3-one (200μg) were added, the buffer was extracted with ethyl acetate and the steroids were separated by t.l.c. on silica gel (mobile phase, chloroform). The 4-cholesten-3-one band was removed and subjected to further t.l.c. under the same conditions to remove all traces of 5-cholesten-3-one, affording 4-[4-^{14}C]cholesten-3-one (5.2μCi).

Preparation of [4β-^{2}*H*]*cholesterol*

[4β-^{2}H]Cholesterol was prepared by the method of Ireland *et al.* (1959). Cholesteryl benzoate (4g) was refluxed with SeO_2 (800mg) in acetic acid/water (100:1, v/v; 50ml). for 5min. Sodium acetate (3.5g) was added, and the 4β-hydroxycholesteryl benzoate (1.03g) that was precipitated was further purified by

dry-column silica-gel chromatography (Smith *et al.*, 1974) and recrystallized (500mg; m.p. 207–208°C). This benzoate (267mg) in pyridine (5ml) was treated with $SOCl_2$ (0.3ml) for 2min at 0°C. The product was extracted into diethyl ether and purified by column chromatography to give 3β-benzyloxy-6β-chloro-4-cholestene (181mg). Reduction of 120mg with $LiAl^2H_4$ (CIBA, Basle, Switzerland) in diethyl ether yielded products (87mg) which were purified by silica-gel t.l.c. (with chloroform as eluent) to give [4β-^{2}H]cholesterol (35.8mg: 77% $^2H_1$); m.p. 146–147°C; n.m.r.* (δ, p.p.m.): 3.52 (multiplet, 3α-H, $J_{2\alpha/3\alpha}$4.5, $J_{2\beta/3\alpha}$10.2, $J_{4\alpha/3\alpha}$4.5); i.r.: C–^{2}H $\nu_{max.}$ 2137cm^{-1}. G.l.c. Kováts (1958) retention index for trimethylsilyl ether was 3120 (cholesterol trimethylsilyl ether, 3120).

Preparation of [4α-^{2}H]cholesterol

4β-Hydroxycholesteryl benzoate (155mg) was stirred overnight with activated MnO_2 (1.5g) in benzene (20ml). T.l.c. of the products (in chloroform/hexane, 1:1, v/v) gave 4-oxocholesteryl benzoate (50mg); m.p. 158–159°C, u.v. $\lambda_{max.}$ 234nm, ε 13600 litre·mol^{-1}·cm^{-1}). The ketone was reduced with NaB^2H_4 (20mg: Prochem, London S.W.19, U.K.) in tetrahydrofuran/methanol (20ml; 1:1, v/v) and 4β-hydroxy[4α-^{2}H]cholesteryl benzoate (6.7mg) was isolated after repeated purifications by t.l.c. Conversion of this ester into 3β-benzyloxy-6β-chloro-4-[4-^{2}H]cholestene followed by reduction with $LiAlH_4$, as above, yielded, after t.l.c., [4α-^{2}H]cholesterol (3.3mg: 81% $^2H_1$); m.p. 147–148°C; n.m.r. (δ, p.p.m.): 3.52 (multiplet, 3α-H, $J_{2\alpha/3\alpha}$ 4.0, $J_{2\beta/3\alpha}$ 10.8, $J_{4\beta/3\alpha}$ 10.8); i.r.: C–^{2}H $\nu_{max.}$ 2177cm^{-1}. G.l.c. Kováts (1958) retention index of trimethylsilyl ether was 3120.

Cholesterol oxidase

Cholesterol oxidase from *Nocardia erythropolis* was from Boehringer (London), London W.5, U.K. The specification was approx. 25 units/mg (Brooks & Smith, 1975) and the sample was found to contain 0.9mg of protein/ml (Lowry *et al.*, 1951). The same vial of enzyme was used for all kinetic experiments, as variations in activity between batches were observed.

General methods

Melting points were determined with a Reichert hot-stage apparatus and are uncorrected. Radioactivity was assayed by liquid-scintillation counting in a Philips PW 4510 scintillation analyser. Samples were dissolved in toluene (10ml) containing 2,5-diphenyloxazole (5g/litre) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.1g/litre). Thin-layer radioscanning was performed with a Panax RTLS-1A instrument. I.r. spectra (of carbon tetrachloride solutions) were obtained with a Perkin–Elmer 580 spectrophotometer by Mrs. F. Lawrie. Proton n.m.r. (100MHz) spectra were measured by Dr. D. S. Rycroft with a Varian XL-100-12 n.m.r. spectrometer fitted with a Varian XL-100 Fourier Transform accessory. Samples were dissolved in [^{2}H]chloroform with tetramethylsilane as an internal standard and with sweep widths of either 1000Hz or 100Hz. Precoated Sil G-25 plates (Macherey–Nagel and Co., Düren, W. Germany) were used for t.l.c. G.l.c. was performed on a Pye 104 gas chromatograph fitted with a column (2.5m×4mm internal diam.) of 1% OV-1 on Gas-Chrom Q (100–120 mesh) at 250°C. Mass spectra were measured by g.l.c.–mass spectrometry by using LKB 9000 or DuPont 21-490F instruments each equipped with columns similar to those used for g.l.c. The carrier gas was helium, the source temperatures were 260–278°C (LKB) and 240–250°C (DuPont), and the electron energy was 70eV. The mass spectrometers were linked on-line to a VG 2035 data system.

All preparative incubations of steroids with cholesterol oxidase were maintained at 25°C and the products extracted with diethyl ether or ethyl acetate.

Determination of K_m and $V_{max.}$

Steroids in propan-2-ol (50μl) were mixed with 2.95ml of 19.5mM-NaH_2PO_4/30.5mM-Na_2HPO_4 buffer, pH7.0, containing 0.25mg of Triton X-100/ml in a cuvette of 1cm light-path maintained at 25°C. Incubations were initiated by the addition of cholesterol oxidase (usually 2.25μg of protein) in buffer (10μl). The rate of Δ^4-3-ketone formation was indicated by the increase in absorption at the appropriate wavelength as monitored with a Cecil Instruments CE272 linear read-out spectrophotometer linked to a Servoscribe recorder. In this system the following parameters were observed: 4-cholesten-3-one, $\lambda_{max.}$ 239nm, ε 12100 litre·mol^{-1}·cm^{-1}; 4-pregnene-3,20-dione, $\lambda_{max.}$ 248nm, ε 15300 litre·mol^{-1}·cm^{-1}; 4-androstene-3,17-dione, $\lambda_{max.}$ 248nm, ε 15400 litre·mol^{-1}·cm^{-1}. Though the detergent Tween 20 was found to interfere less than Triton X-100 in the range 235–250nm, inhibition of cholesterol oxidase was much less with the latter. Increases in absorption in the absence of enzyme were negligible, except with 5-androstene-3,17-dione, for which blank values were subtracted from observed rates. Initial (linear) rates, determined in duplicate for 11–13 substrate concentrations, were used to calculate K_m (μM) and $V_{max.}$ (μmol/min per mg of protein) values. Provisional values were obtained by the graphical method of Eisenthal & Cornish-Bowden (1974). These values were used to obtain more precise values by using a computer program for

* Abbreviation: n.m.r., nuclear magnetic resonance.

a least-squares adjustment of the Michaelis–Menten curve (Wentworth, 1965) on a Digico Micro 16P computer.

Conditions used for incubations

(*a*) *With [4-^{14}C]cholesterol/5-cholesten-3-one.* [4-^{14}C]Cholesterol and 5-cholesten-3-one in propan-2-ol (50μl) were added to 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH7.0 (3ml), and the incubation (at 25°C) was initiated with cholesterol oxidase (5μl).

(*b*) *With [4-^{14}C]4-cholesten-3-one.* [4-^{14}C]4-Cholesten-3-one was dissolved in propan-2-ol (100μl) and mixed with 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH7.0 (6ml). After incubation with enzyme (10μl) for 3h, 4-cholesten-3-one (1mg) and 5-cholesten-3-one (1mg) were added and the steroids extracted.

(*c*) *In buffer containing ^2H_2O.* Buffer containing ^2H_2O was prepared by evaporation of 50mM-NaH_2PO_4/Na_2HPO_4, pH7.0, buffer, desiccating the residue, then adding the appropriate volume of ^2H_2O (99.8%; Fluorochem, Glossop, Derbyshire, U.K.). The pH of reconstituted buffer was checked before use. Small-scale incubations contained steroid (100μg) in propan-2-ol (100μl) with buffer containing ^2H_2O (3ml) and cholesterol oxidase (10μl). Where appropriate, incubations were monitored by the increase in A_{240}. On a larger scale 0.8mg amounts of steroids were incubated in 15ml of buffer prepared with ^2H_2O and containing 500μl of propan-2-ol with 50μl of enzyme. After extraction, the 4-cholesten-3-one was purified by t.l.c. (mobile phase chloroform).

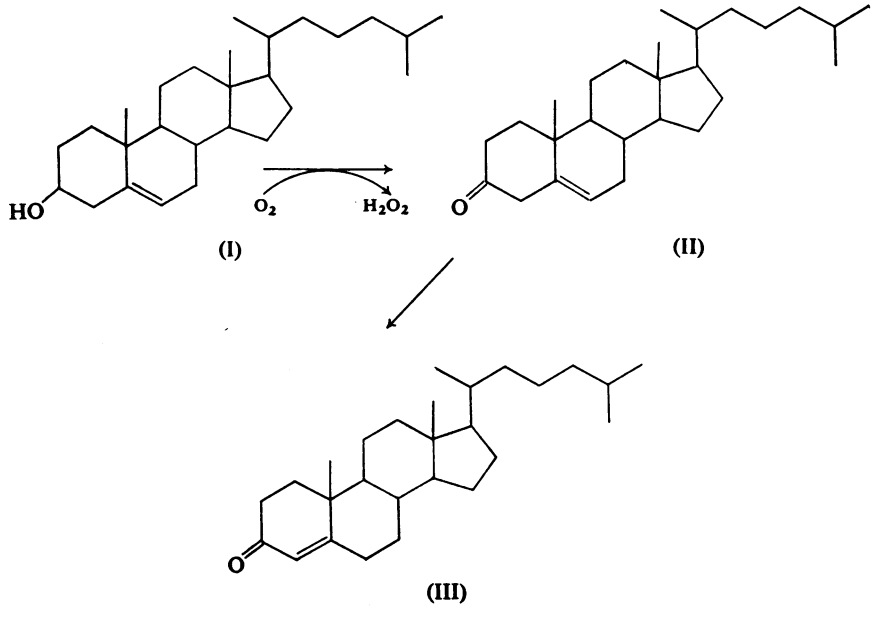
(*d*) *With [4α-^2H]cholesterol and [4β-^2H]cholesterol.* Incubations with ^{2}H-labelled cholesterols were accomplished in 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH7.0, in a manner similar to that described for incubations in buffer prepared with ^2H_2O.

(*e*) *In the presence of acetylenic steroids.* Cholesterol oxidase (10μg of protein, approx. 0.25 unit) was incubated in 0.5ml of 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH7.0, containing 0.25mg of Triton X-100/ml and 10μg (52μM) of either 3β-hydroxy-5,10-seco-19-nor-5-cholestyn-10-one or 5,10-seco-19-nor-5-cholestyne-3,10-dione in propan-2-ol (2μl). A control contained no inhibitor. Samples were withdrawn at timed intervals and the activity of the enzyme for isomerization was tested with 5-cholesten-3-one (50μg, 43μM) in the same manner as described for the kinetic experiments. Oxidase activity was determined, with cholesterol (50μg, 43μM) as the substrate, by measuring the H_2O_2 generated, by using horseradish peroxidase [Boehringer (London), London W.5, U.K.] to couple oxidatively 4-aminoantipyrine and phenol to give a quinone imine absorbing at 500nm (Trinder, 1969; Brooks & Smith 1975).

Results

Formation of 5-cholesten-3-one from cholesterol

Cholesterol oxidase converts cholesterol (I; Scheme 1) into 4-cholesten-3-one (III) with the



Scheme 1. *Conversion of 5-cholesten-3β-ol into 4-cholesten-3-one*

concomitant production of H_2O_2. 5-Cholesten-3-one (II) is probably an intermediate in this process, but no evidence for its presence has been obtained by examining the products of incubation of cholesterol with the enzyme (A. G. Smith, unpublished work). However, a faster rate of isomerization than of oxidation would preclude its accumulation. To trap 5-cholesten-3-one, [4-^{14}C]cholesterol (5×10^5 d.p.m.; 5μg) and 5-cholesten-3-one (100μg) were incubated with cholesterol oxidase and the formation of 4-cholesten-3-one was monitored by the increase in A_{240}. When the isomerization was approximately two-thirds complete, the reaction mixture was shaken with a mixture of water (3ml) and ethyl acetate (6ml) containing, as carriers, cholesterol (100μg), 4-cholesten-3-one (200μg) and 5-cholesten-3-one (450μg). Any [4-^{14}C]5-cholesten-3-one formed during the incubation should have equilibrated, at least partially, with the large excess of unlabelled steroid. A portion (4.6×10^4 d.p.m.) of the extracted steroids was subjected to t.l.c. (mobile phase, chloroform) and examined by thin-layer radioscanning. The major peaks of radioactivity corresponded to 4-cholesten-3-one (R_F 0.53) and cholesterol (R_F 0.26), but a minor peak corresponded to 5-cholesten-3-one (R_F 0.64). Preparative t.l.c. of the remaining portion yielded cholesterol (1.64×10^5 d.p.m.), 4-cholesten-3-one (3.1×10^5 d.p.m.) and 5-cholesten-3-one (1.1×10^4 d.p.m.). The latter fraction was diluted with authentic 5-cholesten-3-one (9mg) and recrystallized twice from methanol with only a small decrease in specific radioactivity (1148, 999 and 1013 d.p.m./mg respectively).

Substrate specificity

The isomerization of 3-oxo Δ^5-steroids by cholesterol oxidases has been reported previously (Kerényi *et al.*, 1975; Smith & Brooks, 1976*a*). To study the influence of the C-17 side chain of 3-oxo Δ^5-steroids on their rate of isomerization by cholesterol oxidase from *N. erythropolis*, the apparent K_m and $V_{max.}$ were determined for 5-cholesten-3-one (II), 5-pregnene-3,20-dione (IV) and 5-androstene-3,17-dione (V). These values were obtained by measuring the rate of increase in absorption in the u.v. caused by the formation of the Δ^4-3-ketone chromophore, and were compared with those determined for the corresponding 3β-hydroxy Δ^5-steroids, i.e. cholesterol, 3β-hydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one respectively (Table 1). The K_m and $V_{max.}$ values for the 3β-hydroxy Δ^5-steroids are measures of the overall rate of oxidation and isomerization. However, as oxidation is apparently the rate-limiting step, the relative values agree closely with those obtained for oxidase activity alone (Brooks & Smith, 1975; Smith & Brooks, 1975, 1976*a*). The $V_{max.}$ values were lower than those measured previously, partly as a result of the use of a lower temperature. The three apparent K_m values were very similar, but cholesterol was oxidized 230 times as fast as 3β-hydroxy-5-androsten-17-one. In contrast, examination of the determinations of apparent K_m and $V_{max.}$ for the isomerization of the 3-oxo Δ^5-steroids (Table 1) revealed that the absence of a C-17 side chain from 5-androstene-3,17-dione greatly increased the apparent K_m value and caused an approximate doubling of the apparent $V_{max.}$ relative to 5-cholesten-3-one.

Stereochemistry

The isomerization of 5-cholesten-3-one (II) to 4-cholesten-3-one (III) necessitates the removal of a 4-hydrogen atom and the insertion of one at position C-6, possibly by intramolecular transfer. [4α-^{2}H]-Cholesterol and [4β-^{2}H]cholesterol (100μg of each) were incubated with cholesterol oxidase to determine the fate of the 4α- and 4β-hydrogen atoms. The products were isolated and the mass spectra of the 4-cholesten-3-ones were obtained by g.l.c.–mass spectrometry. The ^{2}H contents were calculated from the molecular-ion clusters (i.e. *m/e* 384, 385 and 386) by subtraction of contributions from ^{13}C (McLafferty, 1973), and were compared with data for the substrates (Table 2). ^{2}H was partially retained after both incubations, although a higher proportion had been lost from the [4β-^{2}H]cholesterol than from the 4α-isomer. The site of the ^{2}H present in the 4-cholesten-3-one samples was found by examination of the ions of *m/e* 342, 299 and 124 and their respective isotope peaks of *m/e* 343, 300 and 125, which arise from fragmentations involving cleavages of the C-1–C-2, C-3–C-4, C-6–C-7, C-5–C-10 and C-9–C-10 bonds (Shapiro & Djerassi, 1964). In both experiments the isotope peaks were of intensities consistent with the presence of ^{2}H in the same proportions as in the molecular ions; the ^{2}H was therefore located at either position C-4 or C-6 (Shapiro & Djerassi, 1964). To distinguish between these two possibilities, larger amounts (0.8mg) of [4α-^{2}H]cholesterol and [4β-^{2}H]-cholesterol were incubated with cholesterol oxidase, and the 4-cholesten-3-one products were purified by t.l.c. and examined by i.r. spectroscopy. [The bands for the 4-^{2}H, 6α-^{2}H and 6β-^{2}H C–^{2}H stretching vibrations in 4-en-3-ones occur near 2256, 2190 and 2139cm^{-1} respectively (cf. Malhotra & Ringold, 1963, 1964).] Very little absorption corresponding to a 6α-^{2}H band was observed. The relative areas of the other two absorptions, recorded in the linear mode, were determined and are shown in Table 3. Most of the ^{2}H remaining after the incubation of [4α-^{2}H]-cholesterol was still present at position C-4, whereas the ^{2}H remaining from the 4β-isomer had been transferred mainly to the 6β-position. A *cis*-diaxial transfer of hydrogen during isomerization may there-

Table 1. *Comparison of kinetic constants for the action of cholesterol oxidase on 3β-hydroxy Δ⁵-steroids and on 3-oxo Δ⁵-steroids*

Incubations were performed aerobically at 25°C in 2.95ml of 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH7.0, containing 0.25mg of Triton X-100/ml and steroid in propan-2-ol (50μl). Enzyme was added in buffer (10μl). The formation of the 3-oxo Δ⁴-steroid was monitored by the increase in absorption at the appropriate wavelength (239 or 248nm). Apparent kinetic constants were determined as described in the Experimental section. Parameters determined for 3β-hydroxy steroids are measures of both oxidation (rate-limiting step) and isomerization. Results are means ± S.E.M. obtained with 11–13 substrate concentrations in duplicate.

3β-Hydroxy Δ⁵-steroid (range of concns., μM)	K_m (μM)	$V_{max.}$ (μmol/min per mg)	3-Oxo Δ⁵-steroid (range of concns., μM)	K_m (μM)	$V_{max.}$ (μmol/min per mg)
Cholesterol (0.9–43)	5.1 ± 0.1	7.4 ± 0.1	5-Cholesten-3-one (0.9–61)	7.0 ± 0.1	13.9 ± 0.1
3β-Hydroxy-5-pregnen-20-one (1.0–53)	1.7 ± 0.1	3.1 ± 0.2	5-Pregnene-3,20-dione (5.3–106)	12.5 ± 0.3	5.9 ± 0.1
3β-Hydroxy-5-androsten-17-one (1.2–58)	3.6 ± 0.1	0.032 ± 0.001	5-Androstene-3,17-dione (11.7–350)	548 ± 21	23.9 ± 0.8

fore be envisaged (Malhotra & Ringold, 1965). Mass spectrometry showed that some loss of isotope had occurred (Smith & Brooks, 1977*b*) as before, especially in the 4-cholesten-3-one derived from [4β-²H]-cholesterol. To determine whether this loss was due to proton incorporation from the medium, cholesterol and 5-cholesten-3-one were converted into 4-cholesten-3-one by treatment with cholesterol oxidase in 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH7.0, prepared with ^2H_2O. In both cases a moderate incorporation of ²H was observed (Table 2). Incubation of 5-cholesten-3-one on a larger scale (0.8mg) gave a similar incorporation of isotope, as determined by mass spectrometry (Smith & Brooks, 1977*b*), which again was restricted to position C-4 or C-6. I.r. spectroscopy showed that ²H was present mostly at the 6β-position (Table 3); it may have originated from an exchange of hydrogen atoms between the enzyme and the buffer during the process of isomerization. The presence of a small amount of ²H at position C-4 may be explained partly by the slow non-enzymic isomerization that was observed at pH7 (cf. Oleinick & Koritz, 1966). The rate of isomerization of 5-cholesten-3-one (43μM) in buffer containing ^2H_2O was 0.53 of that in protonic buffer, indicating a pronounced isotope effect.

Reversibility

When 4-cholesten-3-one was incubated (in buffer prepared with ^2H_2O) with cholesterol oxidase for 0.25, 3 and 16h, a slow incorporation of ²H was observed (Table 2). Non-enzymic exchange was negligible. (No enzymic incorporation of isotope into 5α-cholestan-3-one occurred under similar conditions.) Reincubation of the 16h sample in protonic buffer resulted in a substantial decrease in the ²H content. Mass spectrometry and n.m.r. of the product from a larger-scale incubation for 16h showed the ²H to be present at position C-6, and i.r. spectroscopy showed it to be at the 6β-position (Table 3). In an attempt to demonstrate the formation of 5-cholesten-3-one, [4-^{14}C]4-cholesten-3-one (5.1 μCi, 82μg) was incubated for 3h with cholesterol oxidase (0.25 unit) and then 4-cholesten-3-one (1mg) and 5-cholesten-3-one (1mg) were added. The steroids were extracted and the 5-cholesten-3-one band was isolated by t.l.c. (mobile phase, chloroform). This fraction (24600 d.p.m.) was diluted with authentic material (34.5mg) to give a specific radioactivity of 255d.p.m./μmol, and recrystallized twice from methanol (90 and 62d.p.m./μmol respectively). The 5-cholesten-3-one was then reduced to cholesterol with $NaBH_4$, purified by t.l.c. (mobile phase, chloroform) and recrystallized from methanol (m.p. 148°C) with some retention of radioactivity (36d.p.m./μmol). Conversion into 5α,6α-epoxycholestan-3β-ol and subsequent recrystallization from

Table 2. *^2H content of labelled 4-cholesten-3-ones formed during incubations with cholesterol oxidase*
Steroids (100μg) dissolved in propan-2-ol (100μl) were mixed with 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH7.0 (3ml), and incubated with cholesterol oxidase (0.2 unit). After extraction of the products with ethyl acetate, mass spectra of the labelled 4-cholesten-3-ones were obtained. The percentages of $^2H_0$-, $^2H_1$- and $^2H_2$-forms were calculated from the molecular-ion clusters by subtraction of contributions from ^{13}C: 4-cholesten-3-one then gave: $^2H_0$, 96%; $^2H_1$, 3%; $^2H_2$, 1%. The ^2H content of 4-cholesten-3-one, after incubation in buffer prepared in ^2H_2O without enzyme for 3h, was: $^2H_0$, 93%; $^2H_1$, 6%; $^2H_2$, 1%.

Substrate	Incubation time (h)	Percentages of labelled forms of 4-cholesten-3-one (%) ^{2}	$^2H_1$	
In protonic buffer				
[4α-^{2}H]Cholesterol*	0.25	48	49	3
[4β-^{2}H]Cholesterol*	0.25	70	29	1
In buffer prepared with ^2H_2O†				
Cholesterol	0.25	57	35	8
5-Cholesten-3-one	0.25	60	31	9
4-Cholesten-3-one	0.25	70	21	9
4-Cholesten-3-one	3	35	60	5
4-Cholesten-3-one	16	10	84	6
(Re-incubated in protonic buffer)	3	67	31	2

* Mass spectrometry of these sterols as the trimethylsilyl ethers gave the following ^2H contents (after subtraction of ^{13}C, ^{29}Si and ^{30}Si contributions): [4α-^{2}H]cholesterol: $^2H_0$, 15%; $^2H_1$, 81%; $^2H_2$, 4%; [4β-^{2}H]cholesterol: $^2H_0$, 20%, $^2H_1$, 77%; $^2H_2$, 3%. Cholesteryl trimethylsilyl ether gave: $^2H_0$, 96%; $^2H_1$, 2%; $^2H_2$, 2%.
† ^2H_2O contained 99.8% ^2H.

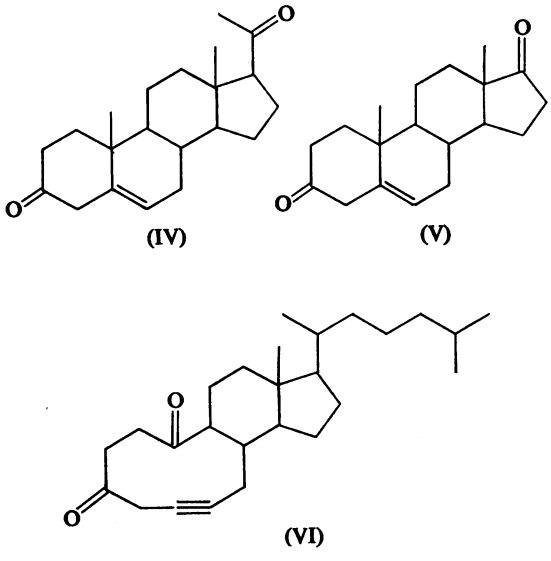
Table 3. *C–^2H stretching vibrations for [^2H]4-cholesten-3-ones formed during incubations with cholesterol oxidase*
Substrates (approx. 0.8mg) in propan-2-ol (500μl) were mixed with 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH7.0 (15ml), and incubated with cholesterol oxidase (0.5 unit). The 4-cholesten-3-one products were extracted with ethyl acetate, purified by t.l.c. and the frequencies of the C–^2H stretching vibrations were then measured. The exact values of ^2H contents as determined by mass spectrometry are reported elsewhere (Smith & Brooks, 1977*b*), but were similar to those shown in Table 2.

Substrate	Incubation time (h)	ν_{C-^2H} bands of 4-cholesten-3-one product (relative areas) 2139cm^{-1} (6β-^{2}H)	2256cm^{-1} (4-^{2}H)
[4α-^{2}H]Cholesterol	1	6	94
[4β-^{2}H]Cholesterol	1	95	5
5-Cholesten-3-one in buffer prepared with ^2H_2O	1	93	7
4-Cholesten-3-one in buffer prepared with ^2H_2O	16	100	0

methanol (Smith & Goad, 1975) gave a specific radioactivity of 40d.p.m./μmol. This yielded provisional evidence for a slow complete reversal of the isomerization.

Inhibition

Batzold & Robinson (1975) have reported the specific and irreversible inhibition of the 3-oxo steroid Δ^4–Δ^5-isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni* by acetylenic seco-steroids. The proposed mechanism involves the conversion of the acetylenic seco-steroid, via enzymic enolization, into an allenic ketone, which reacts with a nucleophilic component of the enzyme (Covey & Robinson, 1976). The effects of 3β-hydroxy-5,10-seco-19-nor-5-cholestyn-10-one and 5,10-seco-19-nor-5-cholestyne-3,10-dione (VI) on the isomerase action of cholesterol oxidase were investigated. The enzyme (0.25 unit) was incubated in 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH7.0, containing 0.25mg of Triton X-100/ml at 25°C together with either seco-steroid (52μM). Portions were withdrawn at intervals, and isomerase activity was tested with 5-cholesten-3-one (43μM). Both compounds progressively inhibited the enzyme (Fig. 1). The 3β-hydroxy compound was less in-



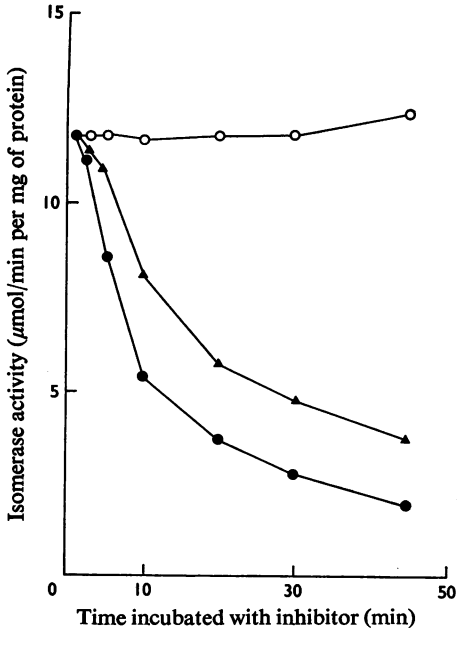


Fig. 1. *Inhibition of the isomerase activity of cholesterol oxidase by 3β-hydroxy-5,10-seco-19-nor-5-cholestyn-10-one* (▲) *and 5,10-seco-19-nor-5-cholestyne-3,10-dione* (●)
Cholesterol oxidase was incubated with the inhibitor (52μM), and samples were removed at intervals and were tested for isomerase activity with 5-cholesten-3-one (○, control).

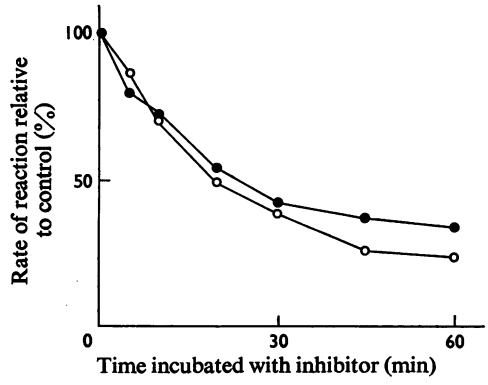


Fig. 2. *Inhibition of the oxidase* (●) *and isomerase* (○) *activities of cholesterol oxidase by 5,10-seco-19-nor-5-cholestyne-3,10-dione*
Cholesterol oxidase was incubated with the inhibitor, and samples were removed at intervals and tested for oxidase and isomerase activity with cholesterol and 5-cholesten-3-one respectively. Results are expressed as percentages of the rate of a control.

hibitory than its 3-oxo analogue, probably because its action depends on prior oxidation. To compare the effects of the 5,10-seco-19-nor-5-cholestyne-3,10-dione on the oxidase and isomerase activities, the incubation of the enzyme with the inhibitor was repeated; portions removed at intervals were tested for both oxidase and isomerase activity (with cholesterol and 5-cholesten-3-one respectively). The results are expressed as percentages of control values (Fig. 2). A steady inhibition of both enzymic functions was observed: the oxidase action was slightly less affected than the isomerase.

Discussion

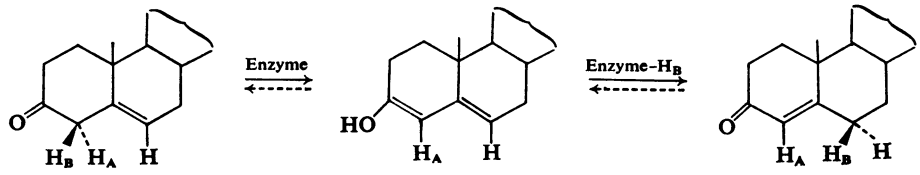
The intermediacy of 5-cholesten-3-one (II) in the oxidation of cholesterol (I) to 4-cholesten-3-one (III) by cholesterol oxidases has been postulated previously (Uwajima *et al.*, 1974; Kerényi *et al.*, 1975; Smith & Brooks, 1976*a*). The trapping of [4-^{14}C]5-cholesten-3-one in a pool of unlabelled ketone after the incubation of [4-^{14}C]cholesterol with *N. erythropolis* cholesterol oxidase demonstrates that the sequence of oxidation followed by isomerization does occur. It is analogous to the 3β-hydroxy Δ^5-steroid dehydrogenase–Δ^4–Δ^5-isomerase systems of mammals, in which the 3-oxo Δ^5-steroid has been assumed to be an intermediate. Edwards *et al.* (1976) identified 5-pregnene-3,20-dione by multiple-ion monitoring during g.l.c.–mass spectrometry of the products of incubation of 3β-hydroxy-5-pregnen-20-one with human placental 3β-hydroxy steroid dehydrogenase–Δ^4–Δ^5-isomerase.

Investigations of the substrate specificity of the

3-oxo steroid Δ^4–Δ^5-isomerase activity of cholesterol oxidase showed that the absence of a C-17 side chain, as illustrated by 5-androstene-3,17-dione, caused a modest increase in the maximum rate of isomerization relative to cholesterol, but considerably increased the K_m. These results are completely different from those obtained for the oxidase reaction (Brooks & Smith, 1975; Smith & Brooks, 1975, 1976*a*). Lack of a side chain at position C-17 in a 3β-hydroxy steroid resulted in a very low rate of oxidation, but had little effect on the K_m. With the oxidase the side chain perhaps serves to orientate the molecule correctly in order to align the 3β-hydroxyl group and the active site for oxidation, whereas during the isomerase reaction the side chain is apparently required to bind the steroid efficiently to the enzyme. The similar rates of isomerization of 5-androstene-3,17-dione and 5-cholesten-3-one by cholesterol oxidase contrast with the extremely slow rate of isomerization of 5-cholesten-3-one by the 3-oxo steroid Δ^4–Δ^5-isomerase of *Pseudomonas testosteroni*. Jones & Wigfield (1968) postulated that the latter effect might be due to the formation of micelles under the conditions used.

The isomerization of 3-oxo Δ^5-steroids by the isomerase from *Ps. testosteroni* has been shown to proceed via an intramolecular transfer of the 4β-hydrogen atom to the 6β-position (Malhotra & Ringold, 1965). To determine the fate of the hydrogen atoms at position C-4 during the conversion of cholesterol into 4-cholesten-3-one (III) by *N. erythropolis* cholesterol oxidase, [4α-^{2}H]- and [4β-^{2}H]-cholesterol were prepared and incubated with the enzyme. Mass spectrometry and i.r. spectroscopy of the products have shown that the 4β-hydrogen atom can be transferred to the 6β-position (Scheme 2), possibly by the mechanism proposed for the 3-oxo steroid Δ^4–Δ^5-isomerase of *Ps. testosteroni* (Wang *et al.*, 1963; Malhotra & Ringold, 1965; Batzold *et al.*, 1976) and for that of ox adrenals (Murota *et al.*, 1971). This postulates enolization of 5-cholesten-3-one (II) with removal of the axial 4β-hydrogen atom followed by re-ketonization and re-protonation at position C-6 (Scheme 2). With cholesterol oxidase, this process was not found to be completely stereospecific, as some ^{2}H was lost from [4α-^{2}H]cholesterol and a small proportion was transferred to position C-6 (Tables 2 and 3). Moreover, both 4α- and 4β-methylcholesterol are slowly converted by the enzyme into 4-methyl-4-cholesten-3-one (Smith & Brooks, 1976*b*). The apparent loss of isotope from [4β-^{2}H]cholesterol can be ascribed to the faster isomerization of the unlabelled species present. Malhotra & Ringold (1965) observed approximately a 30% decrease in ^{2}H content after the isomerization of [4β-^{2}H]5-androstene-3,17-dione to [6β-^{2}H]4-androstene-3,17-dione by the *Ps. testosteroni* enzyme. Further, incubations in buffer prepared with ^2H_2O have shown that exchange of hydrogen with the medium can occur during isomerization of 5-cholesten-3-one by cholesterol oxidase (Tables 2 and 3), possibly during the process of transfer. The enzyme can also catalyse the exchange of hydrogen with the product 4-cholesten-3-one, as ^{2}H was specifically incorporated at the 6β-position (Tables 2 and 3). This observation suggests that reversal of isomerization can take place, at least to the stage of the 3,5-dienol. The incubation of cholesterol oxidase with [4-^{14}C]4-cholesten-3-one, followed by detection of the labelled Δ^5-isomer, provided evidence for reversal of both postulated stages of the isomerase reaction. Wang *et al.* (1963) reported the incorporation of ^{3}H from a medium enriched in ^3H_2O into 4-androstene-3,17-dione, but only by using large amounts of 3-oxo steroid Δ^4–Δ^5-isomerase. Ward & Engel (1966*a*,*b*) have demonstrated the complete reversal of the 3β-hydroxy steroid dehydrogenase–3-oxo steroid Δ^4–Δ^5-isomerase systems of sheep adrenal microsomal fraction and *Ps. testosteroni*, although no reversal of the isomerase reaction alone could be demonstrated.

The proposed mechanism, involving an enolic intermediate, of the isomerization of 3-oxo Δ^5-steroids by *Ps. testosteroni* isomerase, seems to fit all the available evidence (Batzold *et al.*, 1976). The importance of this step in the biosynthesis of mammalian steroid hormones led Batzold & Robinson (1975) to investigate whether the *Ps. testosteroni* enzyme could be specifically inhibited by acetylenic analogues in a manner similar to that first demonstrated by Bloch for β-hydroxydecanoyl thioester dehydratase [D-3-hydroxydecanoyl-(acyl-carrier protein) dehydratase; EC4.2.1.60] (Helmkamp *et al.*, 1968). 5,10-Seco-5-oestryne-3,10,17-trione and 5,10-seco-19-nor-5-pregnyne-3,10,20-trione both irrever-



Scheme 2. *Postulated scheme of isomerization of* 3-oxo Δ^5-*steroids by cholesterol oxidase*

sibly inhibited the *Ps. testosteroni* 3-oxo steroid Δ^4–Δ^5-isomerase. These acetylenic seco-steroids were converted into the allenic ketones, which are considered to act as alkylating agents at the active site of the enzyme (Covey & Robinson, 1976). Our demonstration that 3β-hydroxy-5,10-seco-19-nor-5-cholestyn-10-one and 5,10-seco-19-nor-5-cholestyne-3,10-dione were both potent inhibitors of the isomerase activity of cholesterol oxidase strongly suggests that the mechanism of the isomerization parallels that of the enzyme from *Ps. testosteroni*. The inhibition of both the oxidase and isomerase activities by the acetylenic 3-oxo seco-steroid indicates either that there are two active sites on one protein or that two enzymes are closely associated. Studies of cholesterol oxidases from *Brevibacterium sterolicum* (Uwajima *et al.*, 1974) and *Streptomyces griseocarneus* (Kerényi *et al.*, 1975) are in accord with these conclusions, since no separation of oxidase and isomerase activities was observed during purification; moreover, the former enzyme was obtained in a crystalline state. The low molecular weights (approx. 30000) are also suggestive of single enzymes. In this context, it is worth noting that Ford & Engel (1974) reported a nearly homogeneous preparation of a Δ^4–Δ^5-isomerase–3β-hydroxy steroid dehydrogenase complex from sheep adrenal microsomal fraction.

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