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# CHOLESTEROL CONVERSION TO $\Delta^4$-CHOLESTENONE BY CHOLESTEROL OXIDASE IN POLYPHASIC SYSTEMS : EXTENSION TO THE SELECTIVE OXIDATION OF 7β-HYDROXYCHOLESTEROL

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Abstract : The preparative use of cholesterol oxidase has been extended to polyphasic systems. The enzyme is active in microemulsions with the organic phase composed of mixtures of cyclohexane and chloroform. The kinetic data for oxidation of 7α- and 7β-hydroxycholesterol in microemulsion with the enzyme from *Streptomyces* are similar to those of cholesterol. The cholesterol oxidase is active in the two phase system aqueous buffer - butyl acetate and the preparative enzymic conversion of 7β-hydroxycholesterol to $\Delta^4$-7β-hydroxy-cholestenone was performed in this medium. The enzymic conversion of cholesterol to $\Delta^4$-cholestenone was also performed in two liquid-solid systems, in buffer with cholesterol adsorbed on silica gel and in organic medium with cholesterol oxidase and catalase entrapped in Chromosorb.

Cholesterol oxidase catalyzes the oxidation by dioxygen of the 3β-hydroxyl group of cholesterol and of related steroids to a keto group, and catalyzes the isomerization of the $\Delta^5$ double bond to the conjugated position[1-15] (Fig. 1). Both the oxidase and isomerase activities are on the same protein[1]. The oxidase activity is determined by coupling with the peroxidase[5,16] and the isomerase activity by the 240 nm absorption of $\Delta^4$-cholestenone. The use of this enzyme for preparative purposes became possible when we discovered that catalase could be used to destroy the hydrogen peroxide formed, which otherwise inactivated cholesterol oxidase[17,18,19]. In this article, we wish to report its activity under various heterogeneous conditions.

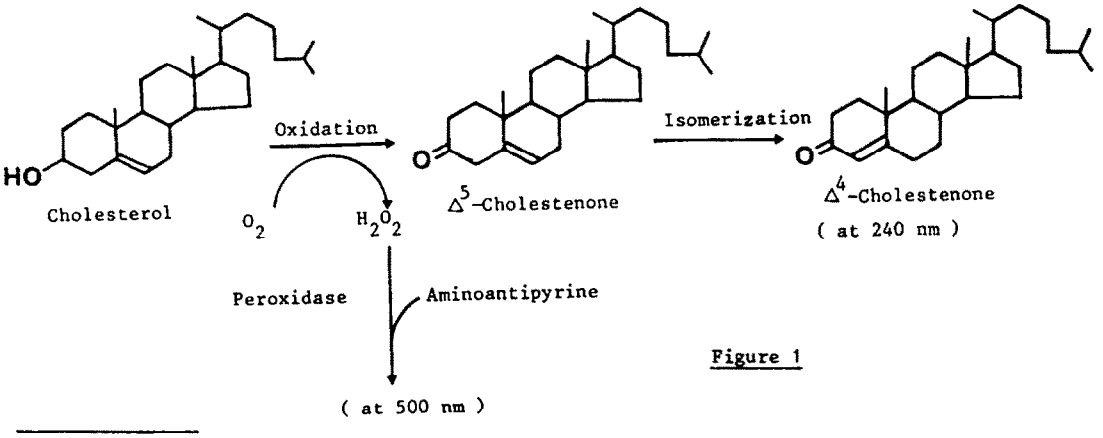


Figure 1

Abbreviations : TES : N-(tris(Hydroxymethyl)methyl-2-aminoethane-sulfonic acid ; CTAB : hexadecyl-trimethylammonium bromide.

RESULTS AND DISCUSSION :

Microemulsions. The advantage of working in microemulsions is that determination of activity by spectroscopic methods is easy to perform, and microemulsions are good models for heterogeneous media. In contrast to normal two phase system, the phase transfer rates are not limiting in microemulsions, so that valid kinetic results may be obtained. For instance the inactivation of cholesterol oxidase by hydrogen peroxide was discovered in such a study. We have used microemulsions made with cyclohexane as organic solvent in which cholesterol is highly soluble[17,19]. However, the 7-hydroxycholesterols were poorly soluble in this microemulsion. We found that a microemulsion in which the 7-hydroxycholesterols were soluble could be prepared with cyclohexane and chloroform mixtures but in a more restricted composition range than with cyclohexane alone.

The substrate specificity of cholesterol oxidase has been studied extensively[1,7,20]. But for steroids hydroxylated at C-7, the oxidation of 7α-hydroxycholesterol with the enzyme from *Nocardia*[21] and of both isomers with the enzyme from *Brevibacterium sterolicum*[2] are the only ones mentioned. We tested the oxidation of both isomers in this microemulsion. 7α- and 7β-hydroxycholesterol were substrates for the enzyme from *Nocardia erythropolis* and *Streptomyces* whereas their oxidation with cholesterol oxidase from *Pseudomonas* was hardly detectable.

In microemulsion Cb-2 (C holds for cationic surfactant, b for n-butanol used as cosurfactant ; for composition see Table 3) the cholesterol oxidase from *Streptomyces* showed stable activity for at least 8 h and the enzyme from *Nocardia* showed a 25 % initial decrease of the activity in 2 h, after which the activity remained stable. The kinetic constants of cholesterol with the enzyme from *Streptomyces* in microemulsion Cb-2 were found to be close to those determined in other microemulsions[17] except for the Michaelis constant which was increased (Table 1). This increase could be due to the higher solubility of cholesterol in the organic phase of microemulsion Cb-2 compared to that of Cb-1. The kinetic constants for the oxidation of 7α- and 7β-hydroxycholesterols with the enzyme from *Streptomyces* were similar enough to those of cholesterol so that a preparative oxidation of these sterols may be envisioned.

Kinetic constants of the oxidation of steroids by cholesterol oxidase from *Streptomyces* in microemulsions Cb-1 and Cb-2, other microemulsions and buffer.

| | Medium | $K_m$ (mM) | $V_{max}$ (mmol.min$^{-1}$.mg$^{-1}$) | |
|---|---|---|---|---|
| 7α-hydroxycholesterol | Cb-1 | 22 | 1.0 | |
| 7β-hydroxycholesterol | " | 13 | 0.47 | |
| cholesterol | " | 18 | 1.5 | |
| cholesterol | Cb-2 | 100 | 1.1 | |
| cholesterol | Other microemulsions* | 28 | 0.9-1.0 | (17) |
| cholesterol | buffer | 0.028 | 3.7 | (17) |
| 7β-hydroxycholesterol | " | 0.0056 | 0.7 | |

* cyclohexane 66 ; buffer 10 ; n-butanol 12 ; Triton X-100 or hexadecyltrimethylammonium bromide 12.

Table 1

Two phase systems. Initially we studied how much the enzyme activity was affected by the presence of an organic phase (Table 2). The oxidation of cholesterol was most effective in toluene, ethyl acetate and butyl acetate. Further studies were carried out in butyl acetate, a good solvent for biphasic reactions with enzymes[22,23]. The oxidase activity of the enzyme from *Streptomyces* decreased with time to 70 % of the initial activity after 48 h. The isomerase activity, in contrast, increased to 160 % of the initial activity after 4 to 8 h, and then decreased to 10 % after 40 h. Both activities followed the same pattern in buffer and buffer saturated with butyl acetate. It is still unknown if both active sites are in proximity or remote from each other. The former is suggested by the fact that both activities are lost by the action of an

inactivating steroid[1,7] and by hydrogen peroxide[18] in a parallel fashion. For preparative purpose, the enzymic oxidation had to be followed by a chemical isomerization (oxalic acid[24]) of some $\Delta^5$-3-ketosteroid still present when the oxidation was completed. This was because the isomerase activity was lost faster than the oxidase activity. The preparative oxidation of 7β-hydroxy cholesterol was performed in the two phase system : buffer-butyl acetate in the presence of catalase. The $\Delta^4$-7β-hydroxycholestenone was obtained in a yield of 92 % after isomerization of the $\Delta^5$-double bond. Cholesterol was converted to a mixture of $\Delta^4$- and $\Delta^5$-cholestenone.

Extent of cholesterol oxidation and percent of $\Delta^4$- and $\Delta^5$-cholestenone produced after 24 h with cholesterol oxidase from *Streptomyces* in two phases systems.

| Organic solvent | % oxidised cholesterol | $\Delta^5$-cholestenone | $\Delta^4$-cholestenone |
|---|---|---|---|
| cyclohexane | 68 | 28 | 40 |
| benzene | 61 | 23 | 38 |
| toluene | 70 | 25 | 45 |
| methylene chloride | 23 | 10 | 13 |
| chloroform | 15 | 7 | 8 |
| carbone tetrachloride | 32 | 13 | 19 |
| diethyl ether | 13 | 6 | 7 |
| ethyl acetate | 75 | 10 | 65 |
| butyl acetate | 77 | 11 | 66 |

Table 2

Solid phase. The use of substrates adsorbed on solids[26,27,28] does not seem to have been reported for enzymic reactions. Cholesterol adsorbed on silica gel was smoothly oxidized by cholesterol oxidase from *Streptomyces*. The isolation of the product was easy and $\Delta^4$-cholestenone was obtained in a yield of 87 %. This procedure may be another effective way to perform enzymic transformations on substrates of low water solubility[29].

Entrapped enzymes. Cholesterol oxidase and catalase were entrapped in Chromosorb and the oxidation of cholesterol in butyl acetate was then performed. $\Delta^4$-Cholestone was isolated in 95 % yield.

Conclusion.

The use of microemulsions has made possible a rather extensive study on cholesterol oxidase. Of great significance is the discovery that the enzyme is rapidly inactived by hydrogen peroxide[17]. As a model for heterogeneous media, the microemulsions have properties which make the study of enzymic reactions easy to perform : transparency, thermodynamic stability and a high rate of exchange between the phases[30,31,32,33]. In the preparative use of microemulsions, the presence of surfactant may hamper the isolation of the product, particularly if polar. However, three other preparative methods to perform enzymic reactions with substrate of low water solubility have been illustrated here : two phase : organic and buffer, substrate adsorbed on a solid suspended in buffer and substrate reacting with an enzyme entrapped on a support in organic solvent. These methods are certainly of a more general use.

EXPERIMENTAL SECTION :

Materials :

Hexadecyltrimethylammonium bromide from Fluka (CTAB) was recrystallized from ethanol, washed with pentane, and dried under vacuum for 3 days. Cyclohexane for UV spectroscopy (Fluka) and 4-amino-antipyrine (Fluka) were used without further purification. n-Butanol (Fluka) was distilled. Other organic solvents were of the highest purity available. The buffer was 50mM TES, pH 7.5. Cholesterol and $\Delta^5$-cholestenone were from Sigma (highest purity). 7α- and 7β-hydroxy-cholesterol were a gift from Dr. LUU Bang of our Institute. Cholesterol oxidase from *Nocardia erythropolis* (25 U/mg), catalase from beef liver, and horse radish peroxidase grade •I were purchased from Boehringer-Mannheim. The cholesterol oxidases from *Pseudomonas* (19U/mg) and

*Streptomyces* species (39U/mg) were purchsed from Sigma. The protein concentration was estimated from information provided by the manufacturer.

Microemulsions :

The composition of the microemulsions is given in % by weight. The microemulsions were prepared at 20°C by adding the various constituents with constant stirring.

| Microemulsion | Cyclohexane | Buffer | CTAB | n-butanol |
|---|---|---|---|---|
| Cb-1 | 66 | 10 | 12 | 12 |
| | Cyclohexane-Chloroform (1:1 vol) | | | |
| Cb-2 | 54 | 10 | 18 | 18 |

Table 3

Activity of cholesterol oxidase :

The enzymes from *Nocardia erythropolis* (0.5mg/ml), *Pseudomonas* (2mg/ml) and *Streptomyces* (5mg/ml) were dissolved in buffer and dialysed. Cholesterol, 7α- or 7β-hydroxycholesterol were dissolved in microemulsion Cb-1. The enzyme solution (6µl) was added to the microemulsion (0.7ml) and the absorption increase was followed at 240nm. For the coupled assay, phenol (120mM), aminoantipyrine (1.3mM) and peroxidase (30U/ml) were added to the microemulsion containing the steroid and the absorption increase was followed at 500nm[5,16,17].

Maximum rate and Michaelis constant of 7α- and 7β-hydroxycholesterol with cholesterol oxidase from *Streptomyces* in microemulsion :

To microemulsion Cb-1 containing cholesterol or 7α- or 7β-hydroxycholesterol (1.7 to 13mM) was added cholesterol oxidase from *Streptomyces* (0.5U/ml). The absorption was followed at 240nm (results in Table 1).

Enzyme activity in microemulsion with chloroform :

The time dependence of the activity of cholesterol oxidase from *Nocardia erythropolis* and *Streptomyces* species was determined in microemulsion Cb-2. The enzyme of *Nocardia* (0.12U/ml) and of *Streptomyces* (1.6U/ml) was incubated in microemulsion Cb-2. At determined times (0 to 8h), an aliquot was taken and added to microemulsion Cb-2 (50µl/ml) containing the cholesterol (0.4M) and the reagents of the coupled assay.

Maximum rate and Michaelis constant of cholesterol in microemulsion with chloroform :

The coupled assay in microemulsion Cb-2 was used with cholesterol oxidase from *Streptomyces* species (0.55U/ml). The cholesterol concentration was varied from 14 to 110µM (results in Table 1).

Maximum velocity and Michaelis constant of 7β-hydroxycholesterol in buffer :

A solution of 7β-hydroxycholesterol (20µM : 25 to 400µl) in buffer containing Triton X-100 (1mg/ml) was added to the same buffer solution to give a final volume of 0.7mL. Cholesterol oxidase from *Streptomyces* (0.16U/ml) was added and the absorption increase at 240nm was followed. The aminoantipyrine and peroxidase solutions were added to the enzyme and the absorption increase at 500nm was followed (results in Table 1).

Activity of cholesterol oxidase in a two phase systems :

A solution of cholesterol (40mM) in organic solvent (2ml) (cyclohexane, benzene, toluene, methylene chloride, chloroform, carbon tetrachloride, diethyl ether, ethyl acetate or butyl acetate) was added to a buffer solution (2ml) containing cholesterol oxidase from *Streptomyces* (5U) and catalase (4000U). After constant stirring under air at 20°C for 24h extraction with cyclohexane was performed. Cholesterol, $\Delta^4$- and $\Delta^5$-cholestenone were separated on thin layer silica gel plates with hexane-ethyl acetate (7:3v/v). After elution of the spots with cyclohexane, the $\Delta^4$-cholestenone was determined by its absorption at 240nm, the $\Delta^5$-cholestenone was isomerized to $\Delta^4$-cholestenone with oxalic acid[24] and then determined as $\Delta^4$-cholestenone as above. The solubility of cholesterol in pentane and dodecane was not sufficient for the present experiments (results see Table 2).

Oxidase and isomerase activity of cholesterol oxidase in butyl acetate saturated buffer :

The buffer was saturated with butyl acetate. The pH did not changed. This saturated buffer was used to prepare all the solutions. The oxidase activity (oxidation of cholesterol with hydrogen peroxide production) was determined by the coupled assay with peroxidase, aminoantipyrine and phenol and the isomerase activity by following the isomerization of $\Delta^5$-cholestenone to $\Delta^4$-cholestenone using the absorption increase at 240nm. The cholesterol and $\Delta^5$-cholestenone solutions were buffer containing Triton X-100 (1mg/ml). The tests were done under the described conditions[17] (results see Discussion).

Enzymic preparative oxidation of cholesterol in a two phase system :

A solution of cholesterol in butyl acetate (200mg/4ml) was stirred under air in presence of a solution of cholesterol oxidase from *Streptomyces* (30U) and catalase (13000U) for 48h at 20°C. The two layers were separated, the butyl acetate solution was dried with magnesium sulfate and evaporated. The residue was chromatographed on silica gel (10g), eluting with a hexane-ethyl acetate gradient from 9:1 to 1:1. $\Delta^4$- and $\Delta^5$-cholestenone were obtained in a yield of 72% and 15% respectively.

Enzymic preparative oxidation of 7β-hydroxycholesterol in a two phase system :

A solution of 7β-hydroxycholesterol in butyl acetate (100mg/6ml) was stirred under air in presence of a buffer solution (6ml) containing cholesterol oxidase from *Streptomyces* (20U) and catalase (13000U) for 48h. The butyl acetate solution was separated, dried with magnesium sulfate and evaporated. The residue was dissolved in ethanol (10ml) and oxalic acid (2mg) was added. After 30min at 60°C, ethanol was removed under partial pressure and the residue was extracted twice with chloroform. The product was then separated on silica gel (10g) with hexane-ethyl acetate gradient. The $\Delta^4$-7β-hydroxycholestenone (m.p. 183-184°C ; Lit.[25] 183-184°C) was obtained in a yield of 92%.

Enzymic oxidation of cholesterol absorbed on silica gel :

A methylene chloride solution of cholesterol (200mg/10ml) was taken to dryness in presence of silica gel (1g : 230-400mesh, Merck). Buffer (10ml) containing the cholesterol oxidase from *Streptomyces* (15U) and catalase (7800U) was added. After 3 days of stirring under $air_4$ the suspension was filtered and the solid washed with ether. After evaporation of ether, the $\Delta^4$-cholestenone was isolated on silica gel (10g) in a yield of 87%.

Cholesterol oxidation with cholesterol oxidase entrapped in Chromosorb :

Chromosorb 101 (80-100mesh, Sigma) was washed with water and buffer and air dried on a glass filter[34]. Cholesterol oxidase from *Streptomyces* (15U) and catalase (13000U) was added to the Chromosorb beads (0.5ml) and the medium was stirred vigorously until complete absorption. The beads were then suspended by stirring under air in a solution of cholesterol in butyl acetate (200mg/5ml) for 3 days. The solution was filtered and the beads washed with butyl acetate. After evaporation of the solvent, the isomerization was done with oxalic acid as above. After evaporation of the ether, $\Delta^4$-cholestenone was obtained in a yield of 95%.

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REFERENCES

(1) Smith, A.G.; Brooks, C.J.W. *Biochem. Soc. Trans.*, 1977, 1088.
(2) Ikawa, S.; Takita, M.; Ogura, M. *J. Biochem.*, 1979, 85, 1447.
(3) Smith, A.G.; Brooks, C.J.W. *Biochem. Soc. Trans.*, 1975, 675.
(4) Uwajima, T.; Yagi, H.; Terada, O. *Agr. Biol. Chem.*, 1974, 38, 1149.
(5) Kerenyi, G.; Szentirmai, A.; Natonek, M. *Acta Microbiol. Acad. Sci. Hung.*, 1975, 22, 487.
(6) Smith, A.G.; Brooks, C.J.W. *J. Steroid Biochem.*, 1976, 7, 705.
(7) Smith, A.G.; Brooks, C.J.W. *Biochem. J.*, 1977, 167, 121.
(8) Sasaki, I.; Gotoh, H.; Yamamoto, R.; Tanaka, H.; Takami, J.; Yamashita, K.; Yamashita, J.; Horio, T. *J. Biochem.*, 1982, 91, 1555.
(9) Keney, W.C.; Singer, T.P.; Fukuyama, M.; Miyake, Y. *J. Biol. Chem.*, 1979, 254, 4689.
(10) McGuinness, E.T.; Brown, H.D.; Chattopadhyay, S.K.; Chen, F. *Biochem. Biophys. Acta*, 1978, 530, 247.
(11) Uwajiwa, T.; Terada, O. *Agr. Biol. Chem.*, 1978, 42, 1453.
(12) Abe, T.; Nihira, T.; Tanaka, A.; Fukui, S. *Biochim. Biophys. Acta*, 1983, 749, 69.
(13) Williams, W.M. *Clin. Chim. Acta*, 1980, 101, 77.
(14) Buckland, B.C.; Dunnill, P.; Lilly, M.D. *Biotechnol. Bioeng.*, 1975, 17, 815.
(15) Fukui, S.; Tanaka, A. *Acta Biotechnol.*, 1981, 1, 339.
(16) Allain, C.C.; Poon, L.S.; Chan, C.S.G.; Richmond, W.; Fu, P.C. *Clin. Chem.*, 1974, 20, 470.
(17) Lee, K.M.; Biellmann, J.F. *Bioorganic Chem.*, 1986, 14, 262.
(18) We have shown by further experiments that both the oxidase and isomerase activities of cholesterol are lost during hydrogen peroxide treatment.
(19) Lee, K.M.; Biellmann, J.F., *Nouveau J. Chimie*, 1986, 10, 675.
(20) 6-Chlorocholesterol (prepared in our laboratory by Dr. C. Thickitt) seems to be a very poor substrate for the cholesterol oxidase from *Nocardia erythropolis*, *Streptomyces* and *Pseudomonas* and caused very slowly the inactivation of these enzymes.
(21) Shimasue, A. *Hiroshima J. Med. Sci.*, 1974, 23, 265.
(22) Cremonesi, P.; Carrea, G.; Ferrara, L.; Antonini, E. *Eur. J. Biochem.*, 1974, 44, 401.
(23) Antonini, E.; Carrea, G.; Cremonesi, P. *Enzyme Microb. Technol.*, 1981, 3, 291.
(24) Oppenauer, R.V. "Organic Syntheses", Vol. 35, Cairns T.L. Ed., John Wiley, 1955, p.43.
(25) Greenhalgh, C.W.; Henbest, N.B.; Jones, E.R.H. *J. Chem. Soc.*, 1952, 2375.
(26) *L'Actualité Chimique*, May 1986 (5), 1.
(27) McKillop, A.; Young, D.W. *Synthesis*, 1979, 401, 481.
(28) Tal, D.; Keinan, E.; Mazur, Y. *J. Amer. Chem. Soc.*, 1979, 101, 502.
(29) We tried the oxidation of solid cholesterol in suspension in buffer containing cholesterol oxidase and catalase. But the oxidation did not proceed to completion, only 30 to 50% of cholesterol was oxidised.
(30) Samama, J.P.; Lee, K.M.; Biellmann, J.F. *Eur. J. Biochem.*, 1987, 163, 609.
(31) Luisi, P.L. *Angew. Chem. Int. Ed. Engl.*, 1985, 24, 439.
(32) Martinek, K.; Levashov, A.V.; Klyachko, N.L.; Khmelnitzki, Y.L.; Berezin, I.V. *Eur. J. Biochem.* 1986, 155, 453.
(33) Waks, M. *Proteins*, 1986, 1, 4.
(34) Cambou, B.; Klibanov, A.M. *J. Am. Chem. Soc.*, 1984, 106, 2687.