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SUBSTRATE SPECIFICITY OF CHOLESTEROL OXIDASE FROM *STREPTOMYCES CINNAMOMEUS*—A MONOLAYER STUDY

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Summary—The substrate specificity of cholesterol oxidase from *Streptomyces cinnamomeus* was examined in oriented sterol monolayers at the air/water interface. Of the cholesterol analogues with structural alterations in the A- or B-ring that were examined, it was observed that 5α-cholestan-3β-ol was oxidized almost as fast as cholesterol itself. When the Δ-5 double bond in cholesterol was instead at the Δ-4 position, the oxidation rate became 3.2-fold slower. A similar reduction in the average oxidation rate was observed when the Δ-5 double bond in cholesterol was instead at the Δ-7 position (5α-cholest-7-en-3β-ol). 5,7-Cholestadien-3β-ol was oxidized 5.1-fold slower compared to cholesterol, whereas 3β-hydroxy-5-cholesten-7-one and 5β-cholestan-3β-ol were not substrates of the enzyme (also verified from the lack of H_2O_2-production). With C(17) side chain analogues of cholesterol, it was observed that the complete lack of the C(17) side chain (5-androsten-3β-ol), or the insertion of an unsaturation at Δ-24 (desmosterol), or even an ethyl group at C(24)(24b-ethyl-5,22-cholestadien-3β-ol) had no appreciable effects on sterol oxidation rate, implying that the enzyme did not recognize the side chain in oriented sterol monolayers. This study has shown that the sterol monolayer system is a good technique to examine sterol/cholesterol oxidase interactions, since both the orientation of the substrate molecules, and the quality of the interface can be mastered.

INTRODUCTION

Cholesterol oxidases (EC 1.1.3.6), or more correctly 3β-hydroxysteroid oxidases, are a group of enzymes found in or secreted by various microorganisms [1–4]. Cholesterol oxidases from several microorganism sources are commercially available. These enzymes have found use both in clinical chemistry and for steroid identification [5, 6], and as tools in cell biology for cholesterol assay and for the subcellular localization of unesterified cholesterol [7–13]. Substrate specificity experiments with cholesterol oxidases from various species have indicated that the 3-hydroxy group in the β-position of the sterol molecule is an absolute substrate requirement to give enzymic oxidation [14–16]. Other studies have indicated that variations in the C(17)-linked side chain of the sterol affects oxidation rates [6, 14]. It has further been reported that sterols with alterations in the ring structure are oxidized slower compared to cholesterol [6, 14–17]. One problem inherent in previous experiments on the substrate specificity of cholesterol oxidases, is the lack of control of the orientation of the substrate molecules relative to the enzyme. The physico–chemical state of the substrate can be expected to affect how efficiently the enzyme can reach its substrate, and therefore this aspect should also be taken into account when interpreting results.

We have recently demonstrated that cholesterol present in monomolecular monolayers at the air/water interface is a good substrate for cholesterol oxidases, both from *Streptomyces* and *Brevibacterium* sp. [18]. With a monolayer system it is possible to control the orientation of the substrate molecules, i.e. the lengthwise axis of the sterol molecule is normal to the plane of the monolayer, and the 3β-hydroxy group is projected into the water phase [18–21]. The activity of cholesterol oxidase (*Streptomyces cinnamomeus*) at the interface of a pure sterol monolayer was shown to be directly proportional to the surface concentration of cholesterol [18]. In mixed monolayers containing phospholipids, other factors such as the lateral surface pressure and the phospholipid acyl chain and head group composition also influence the rate of cholesterol oxidase activity [19, 22–24].

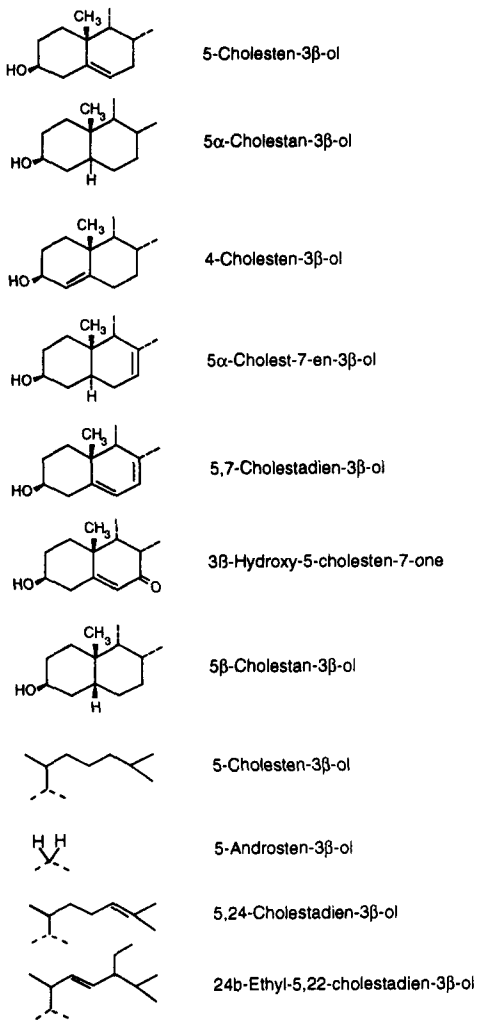

Fig. 1. Molecular schemes of the sterols used in the present study. Only the structures for ring-A and -B are shown. With side chain analogues, only the variation in the side chain at C(17) is shown.

We have now applied the sterol monolayer system to examine the substrate specificities of cholesterol oxidase from *Streptomyces cinnamomeus*. We have examined the cholesterol oxidase susceptibility of cholesterol analogues having structural alterations either in the A- or B-ring of the rigid ring system, or in the more flexible side chain at C(17).

EXPERIMENTAL

Materials

5-Cholesten-3β-ol (cholesterol), 4-cholesten-3β-ol, 5α-cholest-7-en-3β-ol, 5,7-cholestadien-3β-ol (7-dehydrocholesterol), 3β-hydroxy-5-cholesten-7-one (7-ketocholesterol), 5,24-cholestadien,3β-ol (desmosterol), and 24b-ethyl-5,22-cholestadien-3β-ol (stigmasterol) were obtained from Sigma Chemicals (St Louis, MO). These were either over 98% pure by GLC analysis, or were purified by precipitation from ethanol to yield better than 98% purity. 5α-Cholestan-3β-ol, 5β-cholestan-3β-ol, and 5-androsten-3β-ol were obtained from Steraloids (Witon, NH). Buffer salts were of *pro analysis* grade, and the water used was purified by Millex Q to better than 15 MΩ/cm. Cholesterol oxidase (*Streptomyces cinnamomeus*) was purchased from Calbiochem (CA) and was used as delivered.

Lateral surface pressure versus mean molecular area isotherms

Force–area isotherms were determined for pure sterol monolayers with a KSV 3000 Surface Barostat (KSV Instruments, Helsinki). The isotherms were run in a rectangular Teflon trough (450 × 60 mm) on water at 22°C [22]. Stock solution of the lipids were made up in hexane/2-propanol, and were stored desiccated at −20°C. The lipid solution was spread on the buffer, and the monolayer was then allowed to stabilize for 3–5 min before it was compressed at a barrier speed of 10 mm^2/s. Data were sampled every 2 s. At least two different runs were performed with each sterol, and the reproducibility was $> \pm 3\%$.

Oxidation of sterols in monolayer films

The oxidation of sterols in pure monolayers by cholesterol oxidase was determined in a zero-order Teflon trough with Tris buffer (50 mM Tris–HCl, 140 mM NaCl, pH 7.5) [18]. The reaction compartment (2550 mm^2, 30 ml) was magnetically stirred (100 rpm) and thermostated to 22°C. The lipid solution was spread on the buffer surface, and the monolayer was allowed to stabilize for 3–5 min, before it was compressed to 10 mN/m. Constant surface pressure was maintained by compensatory barrier movement (computer controlled) throughout the experiment. After the monolayer had stabilized for 5 min, cholesterol oxidase (16 mU/ml) was added to the reaction compartment. The rate of oxidation of cholesterol in the monolayer was registered (at constant surface pressure) as a backward movement of the barrier due to an oxidation-dependent increase in the monolayer area. Data were sampled every 10 s.

Calculation of enzyme activity

The area over the reaction chamber in the zero-order trough was 2550 mm^2. The mean

Table 1. Oxidation of monolayer sterols by cholesterol oxidase from *Streptomyces* sp.

Sterol species	Mean molecular area at 10 mN/m (A^2)	Oxidation rate relative to 5-cholesten-3β-ol (fold slower)	H_2O_2 production
Ring variation			
5-Cholesten-3β-ol	39.7	1 ± 0.05	Yes
5α-Cholestan-3β-ol	36.9	1.1 ± 0.1	Yes
4-Cholesten-3β-ol	36.0	3.2 ± 0.2	Yes
5α-Cholest-7-en-3β-ol	36.8	3.3 ± 0.2	Yes
5,7-Cholestadien-3β-ol	40.0	5.1 ± 0.3	Yes
3β-Hydroxy-5-cholesten-7-one	38.4	No oxid.	No
5β-Cholestan-3β-ol	40.6	No oxid.	No
Side chain variation			
5-Androsten-3β-ol	37.5	1.1 ± 0.05	Yes
5,24-Cholestadien-3β-ol	40.5	1.1 ± 0.05	Yes
24b-Ethyl-5,22-cholestadien-3β-ol	37.6	1.4 ± 0.1	Yes

The sterols were dissolved in hexane/2-propanol and spread to a monolayer over Tris/NaCl-buffer in a zero-order trough to a lateral surface pressure of 10 mN/m. The oxidation reaction was followed at 22°C with constant surface pressure (10 mN/m), after 16 mU/ml of cholesterol oxidase had been introduced into the subphase of the reaction chamber. The sterol mass that was oxidized (to completion) equaled about 11 nmol and the reaction time for cholesterol was 200 s. Values are averages from 3 different measurements (±SEM). H_2O_2 production indicates whether or not an oxidation reaction could be detected by a fluorimetric assay, as detailed under Experimental.

molecular area determinations of pure monolayers made it possible to calculate the number of sterol molecules that would fit on the surface over the reaction chamber. Since only the surface above the reaction chamber was exposed to cholesterol oxidase, the time needed for the over-all reaction (as determined from the monolayer area expansion, cf. Fig. 2) was equal to the time needed for oxidation of all sterol molecules above the reaction chamber. The oxidation rate observed with cholesterol was fastest (i.e. 3.06×10^{13} molecules oxidized per s), and was set to an arbitrary value of 1.0. All other rates are given relative to the rate observed with cholesterol.

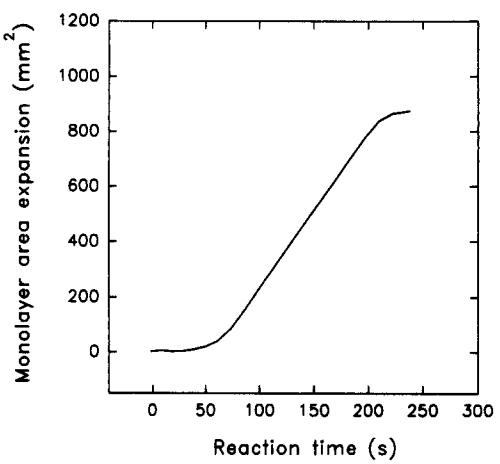

Fig. 2. Oxidation of monolayer cholesterol. This is a representative curve of how data were collected for oxidation of various monolayer sterols. About 20 nmol of cholesterol was spread on Tris buffer, and 16 mU/ml of cholesterol oxidase was introduced into the subphase (30 ml volume) at 22°C. The monolayer expanded (at constant surface pressure, 10 mN/m) due to the enzyme-catalyzed oxidation. The time needed to achieve a post-reaction stable base-line was taken as the total reaction time for the complete oxidation of sterol molecules on the surface of the reaction compartment (about 11 nmol; 2550 mm^2).

Oxidation of sterols in 2-propanol/phosphate buffer

To verify the extent of sterol oxidation from the production of H_2O_2, a fluorimetric cholesterol oxidase assay was employed [7, 19]. The sterols were solubilized in 2-propanol, and were oxidized by cholesterol oxidase in a sodium phosphate buffer (50 mM, pH 7.0) at 22°C. The final reaction mixture (0.405 ml) contained 5 μl 2-propanol (with about 10 nmol sterol) in 400 μl buffer (containing 0.5 U/ml cholesterol oxidase, 250 U/ml horse radish peroxidase, and 0.15 mg/ml *p*-hydroxy phenyl acetic acid). The production of a fluorescent derivative of *p*-hydroxy phenyl acetic acid was detected after 45 min of reaction at 37°C, by recording the relative fluorescence intensity with a Hitachi F-2000 spectrofluorimeter (excitation 325 nm, emission 415 nm).

Molecular modeling

Energy-minimized 3D-models of the sterol were modeled on an Evans-Sutherland computer running the Sybyl Molecular Modeling Software (version 5.41; Tripos Associates, Inc., St Louis, MO). The Tripos internal parameter set was used for the mechanistic energy-minimization procedure.

RESULTS

Force–area isotherms of monolayer sterols

The partial structure of the cholesterol analogues which were used in this study are shown

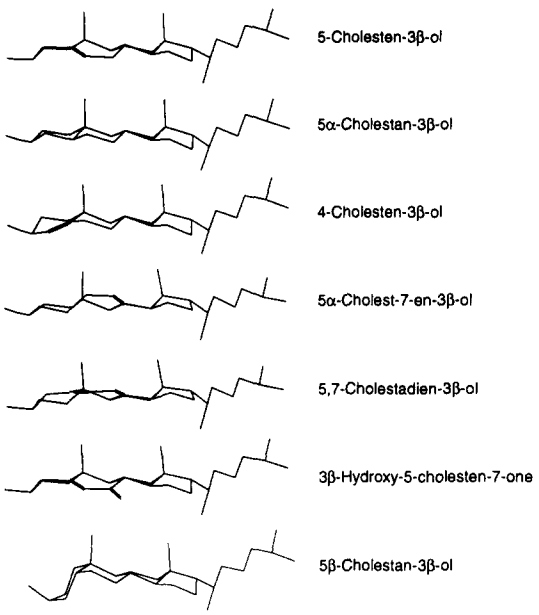

Fig. 3. Energy-minimized models of different cholesterol ring analogues. The molecular structures of the different sterols were energy-minimized, whereupon the molecules were rotated so that C(8) (in ring-B/C) and (11) (ring-C) were in the same plane. Then all hydrogens were removed, and the structure plotted as shown. All bonds shown are between carbons, except the 3β-bond in all structures [between C(3) and an oxygen], and the double at C(7) in 3β-hydroxy-5-cholesten-7-one (carbon–oxygen).

in Fig. 1. Six of the cholesterol analogues had altered structures in either the A- or the B-ring. In addition to the ring analogues, three side chain analogues of cholesterol were examined. A common structure in all sterol was the 3β-hydroxy group, which is an essential requirement for cholesterol oxidase activity [14–16].

The force–area characteristics of these sterols was determined on water at 22°C. Since these sterol analogues have a rigid ring system, the compressed monolayers were solid-like. The observed mean molecular areas at a lateral surface pressure of 10 mN/m were slightly below or around 40 Å^2 for each of the sterol species (Table 1). These values were subsequently used to approximate the number of molecules over the reaction chamber (2550 mm^2) at 10 mN/m, to allow for calculation of the number of molecules oxidized per time unit [18].

Oxidation of sterols by cholesterol oxidase

The basis for the measurement of cholesterol oxidation by cholesterol oxidase in a monolayer system is the larger mean molecular area requirement of the oxidized end-product (4-cholesten-3-one when cholesterol is the substrate) [18]. The enzyme-catalyzed production of a sterol derivative having a larger mean molecular area requirement compared to the substrate molecule will at constant lateral surface pressure lead to a monolayer expansion [18]. A typical time-course for the oxidation of monolayer cholesterol by cholesterol oxidase at a lateral surface pressure of 10 mN/m (at 22°C) is shown in Fig. 2. Similar curves were obtained with all sterol analogues which were oxidized by cholesterol oxidase (not shown). The average oxidation rate observed in a cholesterol monolayer was about 3.06×10^{13} molecules oxidized per s, and this value was arbitrarily set to 1.0, and the average oxidation rate observed in other sterol monolayers were related to this value. The results are presented in Table 1. In the ring analogue series, it was observed that removal of the Δ-5 double bond, to yield 5α-cholestan-3β-ol, had almost no effect on the rate of oxidation. If the Δ-5 double bond was instead positioned at Δ-4, the rate was 3-fold slower compared to the rate observed with cholesterol. A similar 3-fold decrease in oxidation rate was observed if the Δ-5 double bond in cholesterol was instead at the Δ-7 position (to yield 5α-cholest-7-en-3β-ol). 5,7-Cholestadien-3β-ol, which has an additional double bond at Δ-7, was oxidized 5-fold slower than cholesterol. 3β-Hydroxy-5-cholesten-7-one and 5β-cholestan-3β-ol were not substrates for cholesterol oxidase (Table 1).

With side chain analogues, it was consistently observed that modifications in this structure, or even the total absence of the side chain, did not markedly affect the oxidation rate as compared to cholesterol (Table 1).

To verify the cholesterol oxidase susceptibility or resistance of the sterols, we performed an additional assay in which the production of H_2O_2 was determined. With this assay, we confirmed the cholesterol oxidase susceptibility of all sterol analogues, except 3β-hydroxy-5-cholesten-7-one and 5β-cholestan-3β-ol, which appeared to be completely resistant to oxidation (within 45 min at 37°C; Table 1).

DISCUSSION

The novel feature of the present study is the application of the monolayer technique to studies on the substrate specificity of cholesterol oxidase (*Streptomyces*). By having the sterol substrate in the form of a monolayer membrane on the air/water interface, one can control the orientation of sterol molecules relative to the

interface and the enzyme. In comparing the effects of structural alterations in the cholesterol molecule on the oxidation susceptibility, we have used sterol analogues with either side chain alterations, or ring structure alterations.

The results from this study, and from a related study using 5-androsten-3β-ol [19], clearly indicate that cholesterol oxidase does not recognize alterations in the side chain of the sterols. This statement, however, appears to be true only when the sterol substrate is properly oriented, as in a laterally compressed monolayer. Previous studies by others have indicated that a missing side chain, or variable-length side chains, lead to slower oxidation rates both with *Nocardia* and *Streptomyces* oxidases [2, 5, 14]. However, as Smith and Brooks [14] have pointed out, it appears that the C(17) side chain does not greatly affect the degree of sterol binding to the enzyme, but serves to orientate the sterol molecule correctly relative to the enzyme. The present monolayer results clearly show that this interpretation is correct.

Alterations in the ring structure of the sterol molecule is expected to result in more dramatic changes in cholesterol oxidase susceptibility. 5α-Cholestan-3β-ol was in this study oxidized with similar rates as cholesterol, a finding consistent with previous reports on the *Nocardia* enzyme [14]. However, 4-cholesten-3β-ol, 5α-cholest-7-en-3β-ol, and 5,7-cholestadien-3β-ol were all oxidized slower compared to cholesterol. Qualitatively similar results have been obtained with *Nocardia* oxidases [5, 14], although Smith and Brooks reported that 4-cholesten-3β-ol was oxidized with kinetics similar to cholesterol [14].

We have in this study used molecular modeling to derive 3-dimensional, energy-minimized structures of the ring analogues. From these structures (Fig. 3) it can be seen, for example, that 5α-cholestan-3β-ol is structurally very similar to cholesterol (as is the oxidation rate), whereas the A-ring of 5β-cholestan-3β-ol is markedly reoriented away from the plane of the sterol ring system. This A-ring distortion, together with the resulting reorientation of the 3β-hydroxy group, makes this sterol essentially a non-substrate for cholesterol oxidase (Table 1 and [14]). Another interesting comparison can be made regarding the structures presented in Fig. 3: the 3-D structure of 3β-hydroxy-5-cholesten-7-one is very similar to the structure of cholesterol, save for the keto-group extruding at C(7). This 7-keto derivative of cholesterol is not a substrate of cholesterol oxidase (Table 1). This finding suggests that obstructing functional groups at positions in the B-ring will interfere with the successful binding of the sterol molecule to the catalytic site of the enzyme. Sterol analogues which have obstructing methyl groups at position 4 in the A-ring, are also non-substrate molecules for cholesterol oxidases [25].

The crystal structure of cholesterol oxidase from *Brevibacterium sterolicum* was recently reported [26]. This protein is reported to have two domains, an FAD-binding domain, and a steroid-binding domain. The steroid-binding domain is a hydrophobic cavity enclosed by a six-stranded antiparallel β-sheet. The length of the steroid-binding cavity is 11 Å, which is enough to accommodate the tetracyclic steroid ring structure which has a reported length of 10 Å [27]. Although the X-ray structure of an enzyme–steroid complex is still unavailable, it can be assumed that obstructing functional groups in the ring structure of a steroid molecule can prevent its docking into the steroid-binding cavity of the enzyme. This assumption would explain why 3β-hydroxy-5-cholesten-7-one (this study), and 4-methylsterols or lanosterol [25] are not substrates of cholesterol oxidases.

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REFERENCES

1. Stadtman T. C., Cherkes A. and Anfinsen C. B.: Studies on the microbiological degradation of cholesterol. *J. Biol. Chem.* **206** (1954) 511–523.
2. Fukuda H., Kawakami Y. and Nakamura S.: A method to screen anti-cholesterol substances by microbes and a new cholesterol oxidase produced by *Streptomyces violascens*. *Chem. Pharm. Bull.* **21** (1973) 2057–2060.
3. Uwajima T., Yagi H., Nakamura S. and Terada O.: Isolation and crystallization of extracellular 3β-hydroxysteroid oxidase of *Brevibacterium sterolicum* nov. sp. *Agr. Biol. Chem.* **37** (1973) 2345–2350.
4. Smith A. G. and Brooks C. J.: Cholesterol oxidase: properties and applications. *J. Steroid Biochem.* **7** (1976) 705–713.
5. Richmond W.: Preparation and properties of cholesterol oxidase from *Nocardia* sp. and its application to other enzymatic assay of total cholesterol in serum. *Clin. Chem.* **19** (1973) 1350–1356.
6. Smith A. G. and Brooks C. J. W.: Application of cholesterol oxidase in the analysis of steroids. *J. Chromat.* **101** (1974) 373–378.

7. Heider J. G. and Boyett R. L.: The picomole determination of free and total cholesterol in cell in culture. *J. Lipid Res.* **19** (1978) 514–518.
8. Gamble W., Vaughan M., Kruth H. S. and Avigan J.: Procedure for determination of free and total cholesterol in micro- and nanogram amounts suitable for studies with cultured cells. *J. Lipid Res.* **19** (1978) 1068–1070.
9. Gottlieb M. H.: The reactivity of human erythrocyte membrane cholesterol with cholesterol oxidase. *Biochim. Biophys. Acta* **466** (1977) 422–428.
10. Lange Y. and Ramos B. V.: Analysis of the distribution of cholesterol in the intact cell. *J. Biol. Chem.* **258** (1983) 15,130–15,134.
11. Patzer E. J., Wagner R. R. and Barenholz Y.: Cholesterol oxidase as a probe for studying membrane organization. *Nature* **274** (1978) 394–395.
12. Slotte, J. P., Hedström G., Rannström S. and Ekman S.: Effects of sphingomyelin degradation on cell cholesterol oxidizability and steady-state distribution between the cell surface and the cell interior. *Biochim. Biophys. Acta* **985** (1989) 90–96.
13. Thurnhofer H., Gains N., Mutsch B. and Hauser H.: Cholesterol oxidase as a structural probe of biological membranes: its application to brush-border membranes. *Biochim. Biophys. Acta* **856** (1986) 174–181.
14. Smith A. G. and Brooks C. J. W.: Studies on the substrate specificity of cholesterol oxidase from *Nocardia erythropolis* in the oxidation of 3-hydroxy steroids. *Biochem. Soc. Trans.* **3** (1975) 675–677.
15. Kamei T., Takiguchi Y., Suzuki H., Matsuzaki M. and Nakamura S.: Purification of 3β-hydroxysteroid oxidase of *Streptomyces violascens* origin by affinity chromatography on cholesterol. *Chem. Pharm. Bull.* **26** (1978) 2799–2804.
16. Inouye Y., Taguchi K., Fuijii A., Ishimaru K., Nakamura S. and Nomi R.: Purification and characterization of extracellular 3β-hydroxysteroid oxidase produced by *Streptoverticillium cholesterolicum*. *Chem. Pharm. Bull.* **30** (1982) 951–958.
17. Uwajima T., Yagi H. and Terada O.: Properties of crystalline 3β-hydroxysteroid oxidase of *Brevibacterium sterolicum*. *Agr. Biol. Chem.* **38** (1974) 1149–1156.
18. Slotte J. P.: Enzyme-catalyzed oxidation of cholesterol in pure monolayers at the air/water interface. *Biochim. Biophys. Acta* **1123** (1992) 326–333.
19. Slotte J. P.: Cholesterol oxidase susceptibility of cholesterol and 5-androsten-3β-ol in pure sterol monolayers and in mixed monolayers containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. *Biochim. Biophys. Acta* **1124** (1992) 23–28.
20. Birdi K. S. *Lipid and Biopolymer Monolayers at Liquid Interfaces*. Plenum, New York (1989) p. 144.
21. Chapman D., Owens N. F., Phillips M. C. and Walker D. A.: Mixed monolayers of phospholipids and cholesterol. *Biochim. Biophys. Acta* **183** (1969) 458–465.
22. Grönberg L. and Slotte J. P.: Cholesterol oxidase catalyzed oxidation of cholesterol in mixed lipid monolayers: effects of surface pressure and phospholipid composition on catalytic activity. *Biochemistry* **29** (1990) 3173–3178.
23. Slotte J. P. and Grönberg L.: Oxidation of cholesterol in low density and high density lipoproteins by cholesterol oxidase. *J. Lipid Res.* **31** (1990) 2235–2242.
24. Grönberg L., Ruan Z.-S., Bittman R. and Slotte J. P.: Interaction of cholesterol with synthetic sphingomyeline derivatives in mixed monolayers. *Biochemistry* **30** (1991) 10,746–10,754.
25. Wortberg B.: Substrate specificity of cholesteroloxidase from the enzymatic determination of cholesterol. *Z. Lebensm. Unters.-Forsch.* **157** (1975) 333–338.
26. Vrielink A., Lloyd L. F. and Blow D. M.: Crystal structure of cholesterol oxidase from *Brevibacterium sterolicum* refined at 1.8 Å resolution. *J. Molec. Biol.* **219** (1991) 533–554.
27. Craven B. M.: Pseudosymmetry in cholesterol monohydrate. *Acta Crystallogr.* (*Sec. B*) **35** (1979) 1123–1128.