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Review

Cholesterol oxidase: sources, physical properties and analytical applications

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Abstract

Since Flegg (H.M. Flegg, An investigation of the determination of serum cholesterol by an enzymatic method, Ann. Clin. Biochem. 10 (1973) 79–84) and Richmond (W. Richmond, The development of an enzymatic technique for the assay of cholesterol in biological fluids, Scand. J. clin. Lab. Invest. 29 (1972) 25; W. Richmond, Preparation and properties of a bacterial cholesterol oxidase from Nocardia sp. and its application to enzyme assay of total cholesterol in serum, Clinical Chemistry 19 (1973) 1350–1356) first illustrated the suitability of cholesterol oxidase (COD) for the analysis of serum cholesterol, COD has risen to become the most widely used enzyme in clinical laboratories with the exception of glucose oxidase (GOD). The use is widespread because assays incorporating the enzyme are extremely simple, specific, and highly sensitive and thus offer distinct advantages over the Liebermann-Burchard analytical methodologies which employ corrosive reagents and can be prone to unreliable results due to interfering substances such as bilirubin. Individuals can now readily determine their own serum cholesterol levels with a simple disposable test kit. This review discusses COD in some detail and includes the topics: (1) The variety of bacterial sources available; (2) The various extraction/purification protocols utilised in order to obtain protein of sufficient clarification (purity) for use in food/clinical analysis; (3) Significant differences in the properties of the individual enzymes; (4) Substrate specificities of the various enzymes; (5) Examples of biological assays which have employed cholesterol oxidase as an integral part of the analysis, and the various assay protocols; (6) New steroidal products of COD. This review is not a comprehensive description of published work, but is intended to provide an account of recent and current research, and should promote further interest in the application of enzymes to analytical selectivity.

Keywords: Cholesterol oxidase; Review; Cholesterol; Sterol; Substrate specificity; Cholesterol analysis; Sources; Physical properties

Contents

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1. Introduction

1.1. General

The assertion that elevated plasma cholesterol concentrations may lead to an increased chance of developing either atherosclerosis or coronary heart disease (CHD) is now long established [4] and has become a central tenet of the "lipid hypothesis". The need to limit dietary fat and cholesterol intake has been recognised by the UK government in the 1992 Health of The Nation Report [5], and in the United States from the recommendations of the National Research Council [6]. Nevertheless, the role of cholesterol remains controversial. It is not yet clear whether cholesterol is a causal agent for inducing atherosclerosis or if it is the primary exacerbating factor in the formation of foam cells after initial artery wall injury via some other means. The requirement for accurate serum cholesterol determination has as a consequence stimulated a large amount of work on the development of methods for its assay that are fast enough for routine assay, whilst simple and reproducible. Serum cholesterol analysis is now, generally accomplished using a three-enzyme assay and indicator method as originally devised by Richmond et al. [3]. Several versions of this

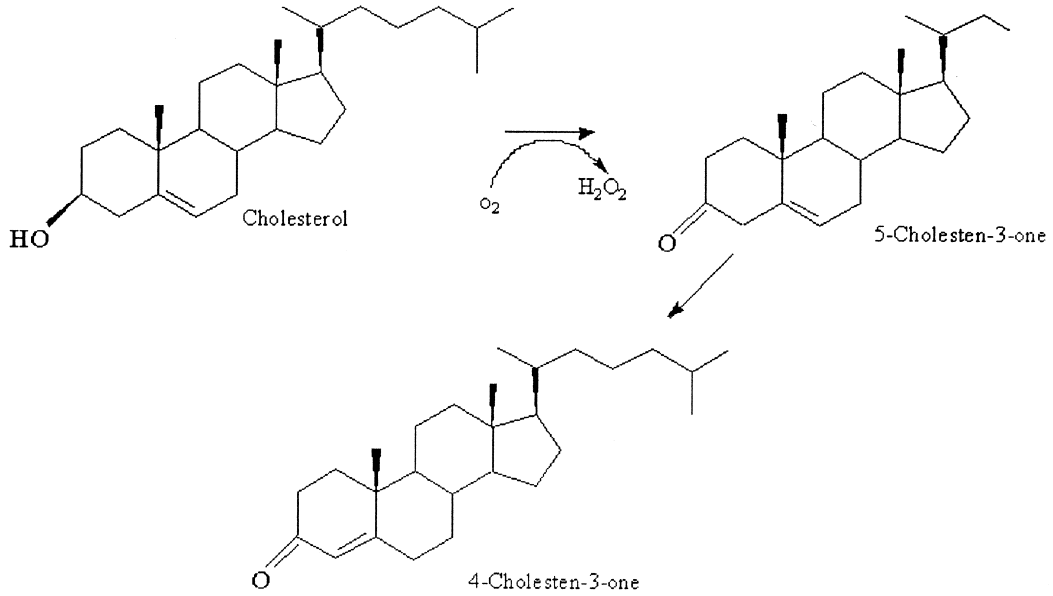

Fig. 1. Mechanism of cholesterol oxidase action.

procedure, based on disposable strip technology are available for use by clinicians [7] and by the general public through home cholesterol test kits [8]. Since ≈70% of the cholesterol present in serum samples is esterified, a typical assay for total serum cholesterol usually begins with the incubation of serum with cholesterol esterase in order to afford free cholesterol, amenable to oxidation with cholesterol oxidase (Fig. 1). A peroxidase enzyme (Fig. 2) subsequently reduces the hydrogen peroxide produced when one molecule of cholesterol is oxidised. Oxidation of an indicator molecule in-turn reactivates the peroxidase and produces a chromogen which when measured facilitates the indirect estimation of total serum cholesterol. Several versions of this procedure exist [9,10]. Separation of these steps has been found to give a better test accuracy than a one-step procedure [10].

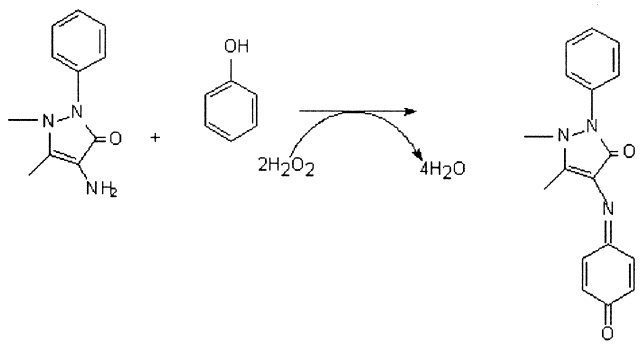

Fig. 2. The assay of hydrogen peroxide by the oxidative coupling of phenol 4-aminoantipyrine catalysed by a peroxidase. Commercial cholesterol assay kits employ both enzyme systems to produce a Schiff's base which is a red dye, and can be determined by adsorption spectrometry.

1.2. Disease states that yield erroneous cholesterol measurements

Although COD catalyses the oxidation, and thus facilitates measurement of plasma/serum cholesterol, it is also active towards a variety of other sterols containing a 3β-hydroxyl group. In several disease states, the concentrations of normally minor plasma sterols are elevated significantly as in the following examples:

1. In patients suffering from Smith–Lemli–Opitz syndrome (SLO), elevated levels of 7-dehydrocholesterol and 8-dehydrocholesterol substantially enhance the test result since they are both substrates of cholesterol oxidase [11].
2. A similar problem is raised in individuals with cerebrotendinous xanthomatosis [12]. In this case, raised levels of 5α-cholestan-3β-ol would augment the "true" serum cholesterol level.

1.3. Mode of action

The enzymes are bifunctional, catalysing not only the oxidation of Δ^5-ene-3β-hydroxysteroids with a *trans* A–B ring junction to the corresponding Δ^5-3-ketosteroid, but also isomerisation to the Δ^4-3-ketosteroid (see Fig. 1; for discussion of the reaction sequence see the review by Smith and Brooks [13]). The isomerisation catalysed by *Brevibacterium sterolicum* has recently been shown to occur via a *cis*-diaxial

intramolecular transfer of protons from the 4β to 6β position [14]. Although the enzymes exhibit a broad range of steroid specificities dependent on bacterial source and/or chemical modification, the presence of a 3β-hydroxyl group is an essential requirement for substrate activity in all cases [15–17]. Stadtman et al. [18] provided the first isolation of the oxidation product using an NAD^+ and $NADP^+$ independent soil mycobacterium. The *Nocardia* enzymes, and others, are simply referred to as oxidases, since their mode of action is dependent only on the presence of molecular oxygen, others are deemed to be true NAD-dependent dehydrogenases [19], e.g. the coupled enzyme 3β-hydroxysteroid:NAD(P) oxidoreductase-3-ketosteroid Δ^4,Δ^5-isomerase (3β-hydroxysteroid dehydrogenase:Δ^5-isomerase) from the mitochondrial fraction of human placenta [20]. In 1976, Smith and Brooks [13] examined the isolation, physical properties, substrate specificity, and the ability of COD to selectively oxidise a variety of different hydroxysteroids. Since 1976, the rate of publications relating to COD has increased to approximately 20 per annum. The increase is driven by a consensus within the medical community of cholesterol's primary role in the pathogenesis of several cardiovascular diseases and consequently the need to produce a method for its routine assay. In addition, the discovery that some oxygenated sterols exhibit biological effects at concentrations at one ten-thousandth the level of that of cholesterol has led to additional work detailing the substrate specificities of several sources of COD [21,22,16,17].

2. Sources of cholesterol oxidase

2.1. General

The taxonomical classification of COD containing microorganisms has developed all through the years since its discovery and some species with originally distinct names have been shown by such methods as DNA profiling to be identical/related. This is discussed in more depth later.

Cholesterol oxidase is produced by several microorganisms that are found in quite differing environments. Turfitt [23–25] was the first to isolate the enzyme from the microbe *Nocardia erythropolis* and to show its effect as an oxidant of cholesterol. Schatz et al. [26] isolated a soil *Mycobacterium*; Stadtman et al. [18] first isolated the product 4-cholesten-3-one from the incubation with a cell free extract of the enzyme (from this *Mycobacterium*). Since then, the enzyme has been found in many microorganisms, notably those listed below.

- *Arthrobacter* [27]
- *Corynebacterium* [28]
- *Nocardia erythropolis* and *Rhodococcus erythropolis* [3,29] (now regarded as the same species)
- *Nocardia rhodochrous* and *Rhodococcus rhodochrous* [30–35]
- *Mycobacterium* [26,36]
- *Pseudomonas* [37]
- *Schizopyllum commune* [38]
- *Brevibacterium sterolicum* [39,40]
- *Streptoverticillium cholesterolicum* [17]
- *Streptomyces violascens* [41–43]
- *Rhodococcus* sp. [44–46]

Rhodococcus is commonly abbreviated to "*R*".

2.2. Species and taxonomic classification

Arthrobacter, *R. equi*, *Nocardia erythropolis*, *Mycobacterium* and *Nocardia rhodochrous* are all intracellular/membrane producers of the enzyme. *Pseudomonas*, *Schizopyllum commune*, *Brevibacterium sterolicum*, *Streptoverticillium cholesterolicum*, and *Streptomyces violascens* express the enzyme into the growth medium. *Schizopyllum commune*, *Brevibacterium sterolicum*, and *Streptoverticillium cholesterolicum* all require FAD, a prosthetic group, for their mode of action. Arima et al. [47] in 1969 surveyed 276 bacterial and 132 actinomycete strains for COD activity, 101 and 123 of which, respectively, were capable of oxidising 20% of cholesterol (0.1%) fed to them. Three strains from *Streptomyces* and one each of *Arthrobacter*, *Bacillus* and *Brevibacterium* were able to oxidise cholesterol at a rate of over 70%. Some strains of the genera *Corynebacterium*, *Rhodococcus*, *Nocardia* and *Mycobacterium* have been the subject of taxonomic study by Ferreira and Tracey [48]. COD from the strain (ATCC 6939) of *Rhodococcus* (*R*) *equi* has been compared with *R. equi* No. 23 from butter, and *R. equi* No. 33 from bacon [44]. *R. equi* No. 23 produced the most extracellular and most active COD. The production of 4-cholesten-3-one was indicated by halo formation on the agar medium. The culture broth of *Rhodococcus* sp. GK1 [45] has been shown to produce an inducible COD. The influence of various carbon sources, including steroids upon the production of *Rhodococcus* sp. GK-1 COD suggested that hexanoic acid was the most effective inducer (of enzyme biosynthesis). Further, the influence of various carbon sources, including steroids, on enzyme biosynthesis was investigated. The carbon sources tested indicated hexanoic acid was the most effective inducer.

R. equi exhibits the highest levels of cholesterol oxidase activity of any of the known COD producing bacteria, but its highly pathogenic nature has ruled out commercial developments [44,46]. Recent data from Sojo et al. [49] suggests that under

appropriate conditions *R. erythropolis* when grown in a simple cholesterol-free glycerol-containing mineral medium cholesterol can show a significantly increased COD activity. The enzyme was initially expressed intracellularly, but latterly into the growth medium. Production of both forms reached an optimum after 1 week and the optimal yield was only obtained when cholesterol was properly solubilised in Tween-80. In addition, variations in the amount of detergent were found not to influence the ratio of cell-bound and extracellular enzyme. During the course of these studies, the authors [49] also examined the physiological features of the production of COD from *R. erythropolis*. Transferral of cholesterol-growing cultures to a medium lacking in cholesterol resulted in the rapid loss of extracellular enzyme, while the intracellular enzyme decreased at a slower rate. Kreit [50] also traced the increase in COD production with *Rhodococcus* sp. GK1 and noted that under the conditions employed, 95% of the initial quantity of sterols (5 g l^{-1}) was consumed in approximately 4 days [50].

Rhodococcus sp. GK1, a strain isolated from soil [50] expresses COD in both, membrane-bound and extracellular forms. Growth of the bacterium in a mineral medium, containing either phytosterols or hexanoate as sole carbon source, was studied and found to be correlated with COD production [50]. Under these conditions, the membrane-bound cholesterol oxidase was induced in large amounts exceeding 100 U g^{-1} dry weight.

Takagi et al. [51] examined the effect of the adsorption of oleic acid onto the cell surface of *Schizopyllum commune* upon cholesterol oxidase production.

Various designations have been given for the four strains of *R. erythropolis*, JCM 2892, JCM 2893, JCM 2894 and JCM 2895 (JCM; Japan Collection of Microorganisms). Originally termed *Corynebacterium hydrocarboclustus* by Iizuka and Komagata [52], was later regarded as a synonym of *Nocardia erythropolis* based upon a study by Komura et al. [53]. Later Goodfellow et al. [54–56], reassigned *N. erythropolis* to the genus *Rhodococcus* (*Rhodococcus erythropolis*). Moreover, *Rhodococcus equi* was transferred from the genus of *Coryneform* bacteria also as a result of rigorous biochemical and DNA tests. (Fuller review of the pathogenicity and epidemiology of this diverse genera is given by Coyle [57] and by McNeil et al [58].) Watanabe et al. [44], while isolating 16 different COD-producing bacterial strains, undertook various morphological, biochemical, physiological and chemotaxonomic studies in order to identify the correct genus classification of 16 bacterial strains isolated from various animal food sources. An inspection of the DNA base composition suggested that these isolated species belonged to the genus *Rhodococcus* Zopf. Several of the isolated enzymes were identified as *Rhodococcus erythropolis* and *Rhodococcus equi* [55] (Goodfellow and Alderson). In addition, different strains of *Rhodococcus* produced varying amounts of enzyme, and some of the isolates were found to produce large amounts of extracellular cholesterol oxidase. Of those identified, *Rhodococcus equi* No. 23 (JCM 6819; Japan Collection of Microorganisms, RIKEN, Wako, Saitama 351-01, Japan) was found to degrade cholesterol without affording identifiable steroidal intermediates, to non-steroidal compounds.

Wilmanska et al. [59] examined the cholesterol oxidase production of four fast growing mycobacteria (*M. fortuitum*, *M. vaccae*, *M. Phlei*, and *M. Smegmatis*) and also *Rhodococcus* sp. IM 58 by means of TLC and Western blot analysis. The strains tested were shown to produce COD intracellularly since culture filtrates possessed no oxidase activity, and only intracellular proteins reacted with antiserum for *Schizopyllum commune*. Western blot experiments revealed high antigenic similarity of the above mentioned proteins to the cholesterol oxidase from *Schizopyllum commune*, and lack of homology to cholesterol oxidase from *Streptomyces* sp. The differences were shown by Southern blot hybridisation to be due to significant differences at the genetic level and not to a single antigenic determinant alteration. Further evidence of the highly polymorphic nature of COD proteins was revealed by the finding that antisera from commercial *Streptomyces* and of *Streptomyces* sp. SA-COO expressed in *S. lividans* were unreactive with homologous intracellular proteins.

2.3. Commercial availability

The cost of cholesterol oxidase remains relatively high due largely to low productivity by the various micro-organisms and to the fact that commercial culture media such as corn-steep liquor require the addition of cholesterol to induce or enhance production [49,50]. To improve the yield, the initial avenue pursued was the enhancement of cell production via alterations of growth medium. Takagi et al. [51] examined the effect of oleic acid adsorption onto the cell surface of *Schizopyllum commune* on yields of cholesterol oxidase. It was found that cholesterol oxidase production depended on the amount of oleic acid in the culture broth, and that it was the level of insoluble oleic acid, rather than the soluble component adsorbed onto the cell, that was directly influencing the formation of cholesterol oxidase. In addition, the optimum extent of COD production was correlated with increases in the rate of agitation.

The gene structures of several sources of COD have been determined and also various attempts have been

made to clone and express COD from various sources with the aim of producing a strain that would be effective for commercial application of enzyme production [40,41,60–75]. Recently, COD has been modified with Polyethylene-glycol (PEG) [76,77] to allow the enzyme to function in organic solvents such as benzene which would normally denature, and thus inactivate the COD protein. Additionally, the COD enzyme has been modified with H_2O_2 by Loubat-Hugel et al. [78].

2.4. Enzyme isolation and purification

2.4.1. General procedures

2.4.1.1. Isolation of enzymes — initial considerations. The procedures involved in the extraction and purification of COD enzymes are on the whole similar to those applied to enzymes in general. However, subtle differences in the extraction and purification procedures are generally required in order to obtain a sufficient yield of active enzyme from each of the COD-producing bacteria to improve yield and/or clarification. The techniques adopted for extraction are largely dependent on the nature of the secretion of the enzyme, i.e. whether membrane bound/intracellular or extracellular. In the latter case, the enzyme is secreted into the growth medium and removal of the cells by centrifugation is often the only step required to obtain a reasonable yield of active enzyme. Extraction of intracellular enzyme is much more difficult because the enzyme must first be removed from within the cell wall by means of cell lysis. Several methods have been developed in order to achieve lysis. Richmond [3] attempted to effect cell lysis initially via mechanical means when extracting enzyme from *Nocardia* sp. Early processes of extraction involved high pressure homogenisation and crushing by means of ball mills. These processes were found to liberate insufficient levels of enzyme and to increase the amount of extraneous protein and other contaminants in the isolated enzyme. Another major disadvantage of mechanical lysis of cells is its tendency to liberate large quantities of nucleic acids, consequently requiring at the earliest stages in the purification procedure an additional step to remove these contaminants.

2.4.1.2. Extraction of cholesterol oxidase via a non-ionic surfactant-based aqueous two-phase system. Albertsson in 1971 [79], was the first to suggest and describe an extraction method based on an aqueous two-phase system of liquid–liquid separation. The primary benefit thereof is the high water content in both phases, resulting in a partitioning which would ensure that protein denaturation of labile biomolecules would be minimised.

A fuller discussion of this form of separation (also referred to as surfactant-mediated phase separation or cloud-point extraction) can be found in a review published by Hinze and Pramauro [80]. The authors describe micellar solutions of polyoxyethylene-type non-ionic detergents which are particularly suited for this purpose, as they exhibit well-characterised phase behaviour. Initially, a single isotropic micellar phase exists, separating upon a change of temperature into two aqueous isotropic phases. One of the resulting two aqueous phases, is richer in detergent (coazervate phase), than the other.

The temperature at which the phase separation occurs is termed the cloud-point, and is governed by the nature of the surfactant, which for polyoxyethylated is temperature-dependent reversible hydration of the polar ethylene oxide head groups. The selective extraction of hydrophobic compounds from aqueous surfactant solutions at increasing temperatures [81] is the result of the formation of lamellar structures in the miscibility gaps of polyoxyethylated fatty alcohol/water systems. The detergent forms micelles in the detergent-depleted phase, and is thought to exist in the form of lamellar stacks in the coazervate phase [82]. In a study by Ramelmeier et al. [83] in 1991, cholesterol oxidases from various bacterial sources (membrane-bound and extracellullar) were studied in Triton X-114R solutions above the cloud point. For a detergent-based aqueous two-phase system, the following factors were investigated for their influence on phase equilibrium and enzyme partitioning temperature: salt, enzyme concentration, source, and pH. The optimised method exhibited a remarkable recovery of the enzyme (over 70 and 90% in the detergent-rich phase for the extracellular and membrane-bound forms, respectively) and a 10- to 20-fold concentration of the enzyme in just one purification step. In addition, the efficacy for the selective partition proteins based on their surface hydrophobicity was illustrated for both hydrophobic and hydrophilic proteins.

2.4.1.3. Enzyme purification. Following extraction, the next stage involves purification or clarification (removal of extraneous proteins), since COD is present in the extraction medium as only a minute proportion of the total protein (particularly so in the case of the intracellularly produced enzyme). Consequently, the first step in the purification process involves the removal of as much of this extraneous protein as possible. Several methods have been applied to effect the first step in the clarification process, and are described below. These general methods are taken from many literature sources and later in this review they are described in detail for each individual enzyme.

2.4.1.4. Fractionation. Ammonium sulphate fractionation is in widespread use as an extremely effective way to carry out the first step in purification [1,39,50,84,85]. This reduces the volume of protein material to a level that can be handled by lower capacity high-resolution techniques such as, ion-exchange chromatography and electrophoresis.

The technique is dependent on the ability of high concentrations of ammonium sulphate ($(NH_4)_2SO_4$) to bind available water molecules and thus to effect solvation of the proteins. For a given set of conditions, the protein precipitates over a characteristic and reasonably narrow range of $(NH_4)_2SO_4$ concentrations, factors generally taken into account include the pH of the medium, the temperature and concentration. Fractionation does have significant drawbacks. Commercial grades of ammonium sulphate often contain heavy metals which cause inactivation of COD. The use of Analar high purity grade $(NH_4)_2SO_4$ however obviates this problem. A further disadvantage is that the precipitated enzyme has to be removed by dialysis, ultrafiltration or desalting of columns before proceeding to the next stage in the purification.

2.4.1.5. Chromatographic purification. Contaminating nucleic acids are commonly removed via a precipitation procedure involving protamine sulphate or alternatively, bound to an anion exchange column (of various sorts, generally DEAE-cellulose), leaving the free enzyme in solution. Where the level of proteinaceous material is sufficiently low, the purity of the enzyme can be improved dramatically via chromatographic means. This is generally the case after ammonium sulphate fractionation. For COD, ion exchange is a widely applied technique. The column normally used is a DEAE cellulose column [2,39,84,86].

Recently, cholesterol affinity columns have led to extraction schemes incorporating this methodology resulting in highly enriched enzyme extracts (×2400) [87].

2.5. Isolation methods for individual enzymes

2.5.1. Nocardia sp.

The first partial purification of the enzyme from *Nocardia erythropolis* was achieved by Flegg [1] and involved precipitation of the cell-free extract in 30% aqueous ammonium sulphate. Richmond [3] showed through a combination of histochemical staining and electron microscopy that isolation techniques incorporating damage to the cell surface offered the greatest degree of release of COD. Richmond found that Triton X-100 (1%) extracted 70% of the total activity of the cell, and that mechanical disruption of the cell in order to obtain COD was ineffective. Previous to Richmonds work, the non-ionic surfactant "Triton X-100" had normally been used in the isolation of mitochondrial enzymes. The crude extract obtained was contaminated with catalase but after purification on a DEAE-cellulose anion-exchange column with a gradient elution procedure involving tri(hydroxylmethyl) aminomethane chloride buffer (pH 8.0), an extract of COD was obtained which exhibited a four-fold increase in activity.

Buckland et al. [86] improved upon this procedure and developed a large scale aerobic process for production of the enzyme. This involved considerably increasing the initial yield of the enzyme through improved bacterial growth. The method depended upon the introduction of cholesterol as an emulsion in Tween 20 to give a final concentration of cholesterol of 2 g/l. The increase in initial yield of the enzyme from the cells was achieved through the induction of a cholesterol emulsion in Tween 20. The extraction procedure was similar to Richmond's [3] early procedure in using a DEAE-cellulose column, but involved a stepwise elution with 50 mM potassium phosphate buffer (pH 7.5) containing 0.5% Triton X-100. The final enzyme was 400× purer although yield was reduced to 26%.

Cheetham et al. [32] in 1980 additionally showed that the enzyme could not be extracted by either changes in pH or ionic strength. However, enzyme was shown to be extracted from cells by treatment with Triton X-100 or trypsin, or to a lesser extent with phosphate buffer, but not by mechanical cell disruption or changes in pH or ionic strength. Cheetham [33] reported that the enzyme preparations extracted from *Nocardia* sp. via these three different extraction procedures possessed similar general enzymatic functions and properties: but he noted differences of the solubility of these enzymes in an aqueous medium. These characteristics were related to differences in the hydrophobic binding domain of the enzyme to the cellular surface, and the hydrophobic nature of the active enzymatic site.

Recently, the use of an aqueous two-phase system with the non-ionic polyoxyethylene detergent "$C_{12}EO_5$" (pentaethyleneglycol mono *n*-dodecyl ether) for direct solubilization and extraction of membrane-bound cholesterol oxidase from an unclarified culture of *Nocardia rhodococcus* was investigated by Minuth et al. [35]. Details of the optimum conditions for the protein extraction from unclarified culture medium were shown on an analytical scale by investigating the influence of detergent and biomass concentration as well as several additional agents for protein solubilisation and stabilisation. Minuth et al. [35] also showed that greater than 90% of the enzyme was taken up by the coazervate phase. This permitted not only a clarification, but a preconcentration (up to four-fold) and a

partial purification (up to five-fold) of the enzyme in only one step.

Although the above method was found to be capable of a COD recovery approaching 100%, the polyoxyethylated surfactants used to attain this extraction level such as, "$C_{12}EO_6$" (hexaethylene-glycol mono *n*-dodecyl ether) and $C_{14}EO_6$ (hexamethyleneglycol mono *n*-tetradecyl ether) are prohibitively expensive and would therefore not be suitable for economic large scale production. Consequently, Minuth et al. [88] investigated the cloud-point extraction performance of an aqueous two-phase system based on a mixed surfactant solution of detergents. Trials with mixed surfactants of technical grade (Dehydol LS4 and Dehydol LT5 and Kn-He 5315/27) were found to be unsuitable for protein extraction. Although, clouding of aqueous solutions of all three surfactant systems occurred at 25–35°C, effective phase separation was not observed at temperatures less than 60°C. The yield of four narrow range ethylene oxide distribution (NRE) surfactants was also studied. Although they were all found to be suitable for extraction, a quaternary system of C_{12}-$C_{18}EO_5$[1] offered a yield almost as high (97%) as the pure $C_{12}EO_5$ surfactant, and had a similar temperature of separation. Solubilisation of the fermentation broth with a detergent to biomass ratio of 1.6 at room temperature for 30 min and phase separation by centrifugation (28°C, 10 min, 400 g) resulted in an initial five-fold enzyme purification and a four-fold preconcentration with 90–95% yield. Detergent recycling was achieved by a simple liquid/liquid partitioning with 2-methylpropanol. Approximately 90–95% of the non-ionic surfactant $C_{12}EO_5$ was extracted into the non-polar phase. The recovery of COD from the aqueous phase was >90%. The organic phase was then re-used as feedstock after distillation to remove solvent from the surfactant. After two recycles, the phase behaviour and yield of the enzyme were re-inspected but found to be essentially unchanged.

Minuth et al. [89] also investigated the cloud point extraction of *Nocardia rhodochrous* using the optimised extraction procedure above on a laboratory scale, and expanded it onto a pilot scale by using commercial disk stack centrifuges. They further examined the influence of operational parameters on the production capacity and the quality of phase separation obtained.

Using such a system followed by ion-exchange chromatography, Kula [90] obtained COD from a whole broth in 75–80% overall yield, with ×160 purification making it suitable for diagnostic and food technology use. It should be noted at this juncture that a cloud point extraction system would be effective for both small and large scale purification of any amphiphilic biomolecule/enzyme.

Rhodococcus erythropolis IMET 7185 [29] (now regarded as the same species as *Nocardia erythropolis*) produces an inducible cholesterol oxidase (COD), which can easily be extracted by treatment of the cells with 0.1% Triton X-100. The yield of the enzyme reported to be 3.3 U/g wet weight from induced cells. A study of the location of COD on intact cells and on ultrathin sections by means of immunogold electron microscopy led to the following conclusions:

1. COD, which is extractable by the detergent, was localized at a distance up to 80 nm above the cell surface. It belongs to a surface layer, which became visible only after lysine/glutaraldehyde treatment and staining with ruthenium red, indicating a high carbohydrate content.
2. Non-extractable COD was found on the cell surface in a shorter distance to the cell wall as well as within the cell wall, in the cytoplasmic membrane and in the peripheral cytoplasm.

In the second case, clusters of gold particles in some places suggest the presence of larger inaccessible amounts of insoluble enzyme.

2.5.2. *Brevibacterium sterolicum*

Uwajima [39] isolated cholesterol oxidase from the source *Brevibacterium sterolicum*, using the following lengthy procedure. The bacterial broth supernatant fluid was concentrated five-fold, a portion precipitated with ammonium sulphate, dialysed, and purified on a DEAE-cellulose column equilibrated with 0.01 M phosphate buffer (pH 7.0) prior to chromatography on a hydroxyapatite and Sephadex G-75 column. The pure crystals of the COD were then crystallised from an ammonium sulphate solution.

2.5.3. *Streptomyces sp.*

The extracellular enzyme isolated from *Strepyomyces violascens* was partially purified by precipitation with saturated ammonium sulphate solution followed by dialysis and lyophilisation [84]. Further purification was achieved on Sephadex G-75, DEAE-cellulose and Sephadex G-200, to give a fraction which showed only one spot during electrophoresis on cellulose acetate. Lartillot et al. [91] purified a fraction of cholesterol oxidase from a *Streptomyces* sp. by means of centrifugation, tangential ultrafiltration, ion-exchange chromatography and gel filtration with a yield of 42% to a specific activity of 54 U I/mg. Its molecular weight determined by SDS-PAGE was 55,000 Da. Its pH at optimal activity was shown to be ca. 7.0.

[1] C_{12}-$C_{18}EO_5$ is the abbreviated term for a narrow range ethylene oxide distribution (NRE) that contains on average five such groups and is inhomogeneous with respect to alkyl chain length.

2.5.4. Rhodococcus sp.

Machangu et al. [85] isolated COD from *Rhodococcus equi* using the following procedure. The purification involved differential ammonium sulphate precipitation, ion-exchange and gel filtration chromatography. This resulted in a purification of 32.8-fold with a yield of 0.3% of COD. The activity of COD was reversibly inhibited by concentration. On sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the COD had a molecular mass (M_r) of 60 kDa.

Rhodococcus sp. GK-1 [50], a strain isolated from soil has been shown to produce both extracellular and intracellular enzymes. In both cases, these can be extracted by cell treatment with Triton X-100, Emulphogen BC-720, Lubrol PX, Lubrol WX, or Brij 76 as described by Kreit et al. [50]. The enzymes were concluded to be solubilised by mixed micelle formation. Of the detergents tested, Triton X-100 released the greatest amount of active enzyme (>90%). The released enzyme in solution was partially purified by precipitation with ammonium sulphate at 60% saturation. The mixture was stirred for 30 min at 0°C. The precipitate was then collected by centrifugation, dissolved in a small volume of phosphate buffer, and dialysed overnight against the same buffer at 2–4°C. The detergent was removed by a batchwise treatment with Amberlite XAD-2 [86]. Evidence for the formation of mixed micelles was provided from both, enzyme filtration on Sephadex G-200 in the presence of Triton X-100, and from the improvement of enzyme activity in the presence of nonionic detergents. In common with other *Nocardia* sp. [3,32], this type of cholesterol oxidase was suggested to be an integral membrane protein and it appeared that the enzyme-active domain was located at the outer surface of the membrane. Substrate catalysis in vivo occurs externally to the cytoplasm. In Kreit's paper [50], a topology model at the membrane level was suggested for cholesterol oxidases of *Rhodococcus* and related taxa.

2.5.5. Pseudomonas sp.

Soil microorganisms producing cholesterol oxidase action have been determined from an incubation broth by the following procedure [87]. Firstly, the soil suspension was spread and embedded in a medium which comprised 1.0% glycerol, 0.5% corn steep liquor, 0.1% KH_2PO_4, 0.1% $NaNO_3$ and 0.05% $MgSO_4$ (pH 7.3) and was solidified with 1.5% agar. The plates were then incubated at 30°C for about 24 h, and bacterial colonies that appeared were replica-plated by toothpick onto the isolation medium plates. The replica plates were then incubated at 30°C for 24 h. In order to select the cholesterol oxidase-producing strains, a colony staining method was used on the agar plates. Filter papers dipped into 0.5% cholesterol, 1.76% 4-aminoantipyrine, 6% phenol and 3000 U/l horseradish peroxidase in 100 mM potassium phosphate buffer (KPB), pH 7.0, were placed on colonies grown on agar medium and incubated at 30°C. The cholesterol oxidase activity of the test colonies was indicated by a red colour, due to the formation of quinoneimine. Strains able to produce the red colour were selected and cultivated at 30°C in 5 ml of liquid isolation medium with constant shaking. The cells were collected by centrifugation, and the culture broth was assayed for extracellular activity of cholesterol oxidase. A bacterial strain, named strain COX629, was selected. The medium used for the purification of the enzyme was a nutrient medium (1.0% peptone, 0.4% yeast extract, 0.1% glucose, 0.5% NaCl, pH 7.2) and the cells were grown at 30°C for 24 h with reciprocal shaking in 3-1 Erlenmeyer flasks containing 1 l medium. The pure enzyme was extracted using the following purification steps. The culture broth was centifuged at 10,000 rpm for 10 min and the clear supernatant fluid obtained, used for purification of the enzyme. The supernatant fluid was directly applied to a DEAE-cellulose column (4 × 40 cm) equilibrated with 10 mM KPB (potassium phosphate buffer), and the column was washed extensively with 500 ml of the same buffer. The cholesterol oxidase was not absorbed on the column under the conditions employed. The active fractions were pooled, and then concentrated by ultrafiltration on a Pellicon Membrane PTGC OLC M2 (Millipore, Bedford, Mass, USA). Commercial cholesterol was recrystallised in 50% ethanol, and the recrystallised cholesterol was used as the adsorbent for the enzyme. The concentrate (508 mg protein per 100 ml) was loaded onto a cholesterol affinity column (1.2 × 30 cm) equilibrated with 10 mM KPB. After washing the column with 100 ml of 10 mM KPB, cholesterol oxidase was eluted with 0.1% Triton X-100, also in 10 mM KPB. To remove cholesterol and Triton X-100 in the enzyme solution, eluates were passed through a Sephadex G-150 column (1.6 × 90 cm) equilibrated with 10 mM KPB. The active fractions were combined, concentrated as above and used for the characterisation of cholesterol oxidase.

Rhee et al. [37] discussed the purification of an active enzyme from the culture broth of a *Pseudomonas* species with a yield of more than 70%. The culture broth (500 ml) of the *Pseudomonas* sp. was centrifuged at 16,000 g for 10 min and the clear supernatant was used for purification of the enzyme. The supernatant fluid was directly loaded onto a column (2.5 × 16 cm) of cholesterylglycine-CM-cellulose (column preparation given), previously equilibrated with 10 mM-potassium phosphate buffer (KPB; pH 7.5) at a flow rate of 1 ml min^{-1}. After washing the column with 200 and 300 ml KPB containing 500 mM-KCl, cholesterol oxidase was

eluted with a linear gradient elution with Triton X-100 (0–0.1%, 600 ml).

2.6. Cloned sources of COD

As noted earlier in this section, several sources of COD have been examined for their cloning and expression into various novel sources. However, most of these produce COD at levels just above or just below that of the wild-type. A notable exception involved the expression of *Brevibacterium sterolicum* into *S. lividans*. The choB gene when expressed into *S. lividans* was shown by Ohta et al. [65] to produce ≈85 times more active enzyme than the wild-type source. The expression of COD from *Brevibacterium sterolicum* (choB gene) in *E. coli* and *S. lividans* was accomplished using various deletion DNA fragments within the 5′-flanking region. Intracellular expression of enzyme in *E. coli* was only detected when the 5′-flanking region was reduced to below 256 base pairs and transcribed by the lac promoter. When choB protein was fused with the NH_2-terminus of the LacZ protein, large amounts of inactive enzyme were produced as "inclusion bodies". In contrast, choB with more than 256 base pairs at the 5′-flanking region was efficiently expressed in *S. lividans*, secreting approximately 85 times as much of the active enzyme (170 U/ml) in comparison with the enzyme from *B. ster* itself into the culture medium. Consequently, Ohta concluded that the promoter of choB lies within 256 base-pairs of the 5′-flanking region, and further that the promoter is readily recognised by the RNA polymerase of *S. lividans*. Amino-acid sequencing showed that the enzymes were identical, with the exception of the *N*-terminus which was found to be six amino acids shorter than *B. ster*. To date, the substrate specificity of cloned COD species has not been studied.

3. Physical properties

3.1. General

The physical properties typically examined include:

1. Operable pH range and optimum pH;
2. Operable temperature range;
3. Reaction solvents/media in which COD is active;
4. The substrate specificity;
5. Molecular weights of the enzymes;
6. Three-dimensional structure (so far determined only for *Brevibacterium sterolicum*);
7. Amino-acid sequences.

In this section, the physical properties are described in general along with their possible implications to steroidal analysis. After this section, the physical properties for each enzyme source are discussed in greater depth individually.

The first four properties have been elucidated for all the commercially isolated sources. The molecular weights have been determined for all sources from chromatographic data (gel permeation/size exclusion) and are possibly imprecise. This is perhaps due to extraction of extraneous protein in addition to active protein or the formation of mixed micelles. COD is generally optimally active at a temperature of 37°C and a pH of 7.0 in an aqueous medium (detailed in the sections for each individual source) — although the activities vary considerably, some often offering better thermal stabilities and/or broader pH ranges (see especially discussion on *Streptomyces* sp. physical properties in Section 3.5).

It is important to note that the physical properties of COD will play a major role in determining different substrate specificities; a discussion of which is given in Section 4. Investigators in this area originally examined specificity in a "one-pot" or uniphasic medium. Only recently have specificities been examined in the more rigorous confines of a saturated monolayer [21]. The advantages of such a system are that the sterol substrate can be specifically orientated for entrance to the active site. Examinations of the varying substrate specificity of the enzymes will be useful for analysis purposes. For example, in cases where there may be only one capillary column available, the sterols can be modified and run on the same column but as the Δ^4-3-one derivative [92] (see Section 5).

Perhaps the primary advantage of amino-acid sequencing will lie in the application of the sequence for the development of modified enzymes capable of operating in a variety of conditions e.g. pH, organic solvents, thermal stability and specificities. Currently, amino-acid sequencing has been applied to COD from only a few sources. Amino-acid sequences and structural elucidation may uncover whether each COD functions are similar in the nature of oxidation and isomerisation steps and may also account for differences in specificity.

3.2. Nocardia sp.

The *Nocardia* sp. enzymes exhibit a broad pH optimum around 7.0 and a temperature optimum of 32°C [13]. Although the enzyme is thermally stable at temperatures as high as 50°C, the activity of the enzyme is held best when stored at 5°C [13]. As noted earlier (sources section 2.5.1: *Nocardia* sp.), three forms of the *N. Rhodochrous* enzyme have been isolated [32,33]. Cheetham et al. [32,33] described the following observations. Although the specificity, optimal pH, temperature-activity profiles and inhibition characteristics are the same, the trypsin, buffer, and Triton-extracted

enzymes differed quite substantially in several key physical properties. It was observed that the K_m for cholesterol of 55, 45, and 69 ± 0.3 mM with maximal activities at 0, 0.005 and 0.15% concentration of Triton X-100, respectively. In comparison, a value of 24 mM was obtained with the cell-bound COD. Additionally, two pieces of evidence suggested the formation of mixed micelles. Firstly, the Triton-extracted enzyme displayed surface-dilution kinetic behaviour. Secondly, gel filtration on Sephadex G-200, in the presence of 0.5% (v/v) detergent, resolved Triton-extracted enzyme into three distinct peaks. Chromatography of the Triton- or buffer-extracted enzyme caused both enzymes to aggregate and to be eluted in the void volume. In all cases, organic solvents present (even at very low concentrations) in the reaction medium resulted in a marked increase in reaction rate. Incubation of the enzyme in the presence of isopropanol at concentrations up to 5% (v/v) enhanced reactivity at first but reduced it thereafter. Trypsin-extracted enzyme lost activity even at low concentrations [32,33]. Activity at high concentrations (at and above 25%) was much reduced and was attributed to the disaggregation of mixed micelles as first discovered by Becher in 1965 [93]. The bimodal (amphipathic) nature was evident in the inability of the trypsin-extracted enzyme to be adsorbed on an Amberlite IRC-50 column at pH 7.0, in contrast to either the Triton or buffer enzymes — providing further confirmation that the enzyme extracted with trypsin lacks the hydrophobic character evident in the latter (buffer and Triton extracted). The behaviour a particular enzyme displayed was found to be interconvertible under a variety of treatments.

3.3. Rhodococcus sp. GK1

This strain of enzyme has been shown to be extracted in both extracellular and membrane-bound forms. In common with *Nocardia rhodochrous* [33] and several other related strains found by Halpern [94] and Johnson and Somkuti [95], this enzyme has been extracted as mixed micelles (based on the low cmc of the surfactants, activation of the enzyme in the presence of the surfactant and filtration characteristics on Sephadex G-200). The enzyme was shown to be bimodal amphipathic and it was suggested that it could be assigned to the class of 'stalked intrinsic membrane proteins', a group that includes several intestinal and renal brush border enzymes, such as γ-glutamyltranspeptidase from rat kidney [96] and sucrase-isomaltase complex from rabbit small intestine [97]. However, Kreit et al. found that the substrate specificity of the extracellular [45] and intracellular [50] enzymes of species GK1 was similar. It should be noted that three significant differences were noted by Kreit for species GK1 [45,50].

1. Both enzyme types have an optimal pH region: the membrane-bound type (6.0–8.2) is broader than that of the extracellular enzyme (7.0–7.5).
2. The membrane-bound enzyme is much more hydrophobic than the extracellular type.
3. The gel filtration data reflected a difference between the molecular masses of both enzymes: the membrane-bound form is higher than that of the extracellular enzyme. The predicted enzyme weights are 80 and 40 kDa, respectively, for the membrane-bound and extracellular enzymes [50].

3.4. Brevibacterium sterolicum

3.4.1. General

Uwajima et al. [39] in 1973 studied the physical properties of COD obtained from *B. sterolicum* (*B. ster*) in great detail. From a sedimentation coefficient of 3.3 S obtained in water at 20°C, a molecular weight of 32,500 Da was calculated. In contrast, Fujishoro et al. [73] from amino-acid sequencing results suggested later that the mature enzyme consists of 507 amino acids with a preceding 45-amino-acid signal sequence yielding a predicted M_r of 55 kDa. (Sephadex G-75 gel filtration indicated a similar molecular weight). Uwajima et al. [39] found that this enzyme contains a flavin adenosine dinucleotide (FAD) prosthetic group for the oxidase apoprotein. The prosthetic group, which was identified from its absorption spectrum and by comparative paper chromatography, was released upon heat treatment at 100°C for 3 min. Metal ions and chelating agents were generally not inhibitory, with the exception of silver and mercury compounds — although their effect was found to be negligible in the presence of glutathione. The enzyme from this source is very stable, possessing a broad active pH around 7.5. Maximal activity can be retained over 30 min at 50°C in 0.1 M phosphate buffer of pH 7.0, but the enzyme was also stable for 30 min at 37°C over the pH range 4–10 [39].

3.4.2. Surface chemistry/dynamics

Slotte [98] has examined the kinetics of the oxidation of a cholesterol monolayer (with *B. sterolicum*) at various surface pressures. In order to do this, the mean molecular area versus lateral surface pressure isotherms for monolayers containing cholesterol, 4-cholesten-3-one (cholestenone), and binary mixtures of the two were first determined. Ranges of lateral surface pressures were examined in each case. At every pressure, cholestenone had a larger mean molecular area requirement than cholesterol.

Binary mixtures of cholesterol and cholestenone exhibited a non-additive mean molecular area suggested non-ideal mixing of the sterols, since the

observed mean molecular area for mixtures was less than would be expected based upon ideal mixing. When the lateral collapse pressure was examined for the mixed sterol monolayers, a lower value than expected was found. The pressure was found to correlate directly with the mole fraction of cholestenone in the monolayer, suggesting that cholesterol and cholestenone were however completely miscible in the mixed monolayer. Armed with these results, investigators were able to use a pure cholesterol monolayer to examine the effect of surface pressure upon the kinetics of cholesterol oxidase-catalysed (*Brevibacterium* sp.) oxidation of cholesterol to cholestenone at different lateral surface pressures at 22°C. Since there was a difference in the mean molecular area requirements of cholesterol and cholestenone, monolayer area changes (at constant lateral surface pressure) could then be converted into average reaction rates. The following points are noteworthy.

1. It was observed that the average catalytic activity of cholesterol oxidase increased linearly with increased lateral surface pressure in the range of 1–20 mN/m. In addition, the enzyme was capable of oxidizing cholesterol in monolayers with a lateral surface pressure close to the collapse pressure of cholesterol monolayers (for a collapse pressure 45 mN/m; oxidation was observed at 40 mN/m).
2. The absorption of cholesterol oxidase to an inert sterol monolayer film at low surface pressures (around 9 mN/m) although marginal, was clearly detectable at very low (0.5–4 mN/m) lateral surface pressures, suggesting the enzyme did not penetrate deeply into the monolayer in order to reach the 3β-hydroxy group of cholesterol. (This interpretation was further supported by the finding that a maximally compressed cholesterol monolayer (40 mN/m) was readily susceptible to enzyme-catalysed oxidation).

Slotte [98] thereby concluded that cholesterol oxidase from *B. ster* is capable of oxidizing cholesterol in laterally expanded monolayers as well as in tightly packed monolayers, where the lateral surface pressure is close to the collapse pressure. The kinetic results suggested that the rate-limiting step in the overall process was the substrate availability per surface area (or surface concentration) at the water/lipid interface.

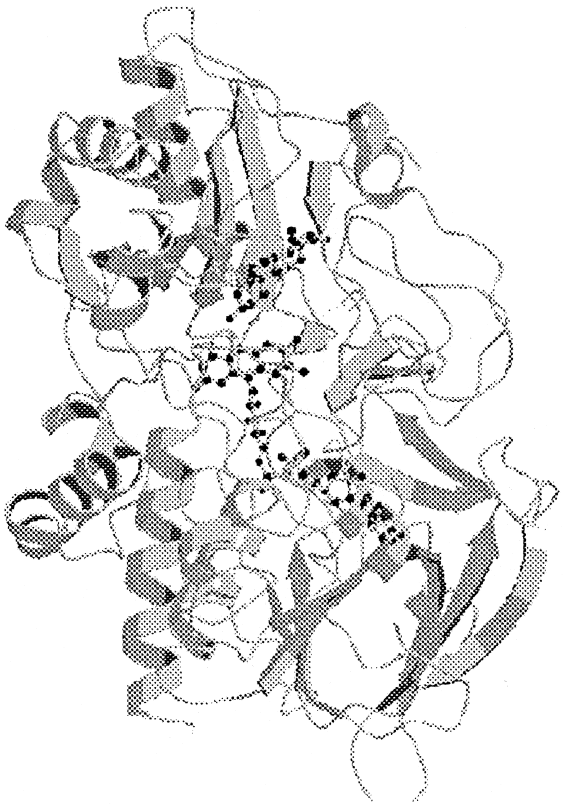

Fig. 3. An illustration of the structure of cholesterol oxidase showing the secondary protein structural elements. The atoms of the steroid and FAD cofactor are represented by black disks. The bound steroid, dehydroisoandrosterone, is situated towards the top of the enzyme, and the FAD cofactor site (below the steroid) is near the centre of the enzyme. Reproduced by kind permission of Professor Alice Vrielink [100].

3.4.3. Crystalline structure in an unbound and substrate bound state

The Vrielink group describes the crystalline structure of *B. ster* in remarkable detail for both, an unbound [99] and substrate bound state [100]. Fig. 3 illustrates the substrate bound form of the enzyme and illustrates the substrate and active site interaction.

In the free state the crystal structure [99] was determined and refined to a 1.8 Å resolution using the method of isomorphous replacement. The following is a summary of the salient points, since a full discussion of the structure and the background to its determination is complex and beyond the scope of this review. The refined model includes 492 amino-acid residues, the FAD prosthetic group and 453 solvent molecules. The crystallographic R-factor is 15.3% for all reflections between 10.0 and 1.8 Å resolution. The structure comprises of two distinct domains: a FAD-binding domain and a steroid-binding domain. The FAD-binding domain consists of three non-continuous segments of sequence, containing the N and the C termini, and comprises "a six-stranded beta-sheet sandwiched between a four-stranded beta-sheet and three alpha-helices" [99], a topology which is shared by other FAD-binding proteins. The steroid-binding domain consists of two non-continuous segments of sequence and contains a six-stranded anti-parallel beta-sheet which can be considered as a "roof" to the active-site cavity. "The active site lies at the interface of the two

domains, in a large cavity filled with a well-ordered lattice of 13 solvent molecules. The flavin ring system of FAD lies on the "floor" of the cavity with N-5 of the ring system exposed. The ring system is twisted from a planar conformation by an angle of approximately 17°, allowing hydrogen-bond interactions between the protein and the pyrimidine ring of FAD. The amino-acid residues that line the active site are predominantly hydrophobic along the side of the cavity nearest the benzene ring of the flavin ring system, and are more hydrophilic on the opposite side near the pyrimidine ring. The cavity is buried inside the protein molecule, but three hydrophobic loops at the surface of the molecule show relatively high temperature factors, suggesting a flexible region that may form a possible path by which the substrate could enter the cavity. The active-site cavity contains one charged residue, Glu361, for which the side-chain electron density suggests a high degree of mobility for the side chain. This residue is appropriately positioned to act as the proton acceptor in the proposed mechanism for the isomerisation step [99]".

As alluded to earlier, the bound form of COD with dehydroisoandrosterone (DHIA) has been determined by Li et al. [100] and is illustrated in Fig. 3. DHIA is shown in an internal "hollow" within the protein structure which is completely enclosed from solvent when either substrate is bound or unbound. In the absence of the steroid, this "hollow" contains a "lattice" of H_2O molecules. It was further shown that the conformation of the protein changes to allow the substrate to fit within the enzyme "hollow".

The flavin prosthetic group and a water molecule are hydrogen bonded to the hydroxyl groups of the steroid. This feature shows a family resemblence to the glucose oxidase enzyme of the GMC (glucose–methanol–choline) type [101].

The nature of the mechanism of flavin-assisted oxidation has been the subject of speculation based upon the derived structures [99,100], and is postulated to occur by one of two possible mechanisms: a radical-mediated mechanism or a hydride-transfer mechanism. Medina et al. [102] examined the production of the flavin semiquinone since during the radical mechanism this moiety should be generated, although the mechanisms for oxidation of the sterol and reduction of the flavin were not conclusively established. The results provided further evidence for the previous conclusion of Li et al. [100] that *Brevibacterium Sterolicum* COD shares significant structural homology, and possibly a similar oxidation mechanism with enzymes in the GMC oxidoreductase family [101].

Recently, the isomerisation reaction catalysed by *B. ster* has been shown to occur via a stereospecific proton transfer from the 4β carbon to the 6β carbon to form 4-cholestene-3-one using deuterated and non-deuterated substrates [14]. On the basis of the results, one active site base, positioned over the β-face, was suggested as being responsible for isomerisation. On the basis of the structural determination detailed by Vrielink et al. [99], glutamate-361 residue was suggested by the authors to fulfil this function [14].

Furthermore, Medina et al. [102] described the effects of the reduction of the flavin of COD from *B. ster*, at pH values above 7 in the presence of EDTA, with either sodium dithionite or light irradiation (in the presence or absence of deazariboflavin) was shown to occur through a stable intermediate state. The spectrum of the intermediate was indicative of a flavin anionic semiquinone. The rate and extent of reduction were pH dependent. Reduction by these agents at pH values of 6.5 (or below or at the pH's when dehydroisoandrosterone, a protein substrate analogue, was used as reductant) did not produce an intermediate and furthermore, no intermediate radical was detected during the reoxidation process. Treatment of cholesterol oxidase semiquinone with dehyroepiandrosterone did not convert the semiquinone to the fully reduced state. The absorption coefficient of oxidised cholesterol oxidase at 470 nm is 10^3 M^{-1} cm^{-1}.

3.4.4. ESR and ENDOR spectroscopy

Medina et al. [102] also examined the ESR (electron spin resonance) and ENDOR (Electron nuclear double resonance) spectra of the flavin semiquinone intermediate. The ESR signal of *B. ster*. COD semiquinone was centred on $g = 2.004$ with a linewidth of 1.48 mT in both H_2O and D_2O environments. Upon the addition of DHIA, the linewidth decreased to 1.43 mT. Measurements of linewidths were similar at either X or S band frequencies indicating that line broadening was due to hyperfine interactions. ENDOR spectroscopy of cholesterol oxidase semiquinone provided further information about the nature of the interactions of the flavin radical with protons. "A group of signals, with couplings of 9–12 MHz, was attributed to protons on 8-CH_3 ($A_{iso} = 10.9$ MHz) and on C_6 ($A_{iso} = 9$ MHz) of the flavin ring. In the presence of D_2O, no change in hyperfine coupling constant was detected. The 8-CH_3 decreased by 0.98 MHz when the ENDOR spectrum was studied in the presence of DEP (also 9.92 MHz). A second group of signals was observed with hyperfine couplings of less than 2.5 MHz. Some of these weak couplings disappeared when the protein was transferred to D_2O, or when the substrate, dehydroepiandrosterone was present. The signals are attributed to displaced water protons or exchangeable protons from amino-acid residues on the protein near the flavin binding site, involved in substrate stabilisation (c.f. active site determined by Vrielink et al. [99]). The decrease in the isotropic hyperfine coupling constant of the 8-CH_3 protons after substrate binding

may be ascribed to an electron withdrawing effect of the hydroxyl group upon the flavin ring system [102]''. Medina et al. [102] propose that substrate binding may polarise electron density onto the pyrimidine ring, and in doing so, decrease electron spin density on the benzene ring.

3.5. Streptomyces sp.

Tomioka et al. [103] undertook the first thorough investigation of a *Streptomyces* sp. in 1976. Listed below is a summary of the physical properties found for their enzyme, *Streptomyces violascens*.

1. Molecular weight of 30,000 (calculated from the elution volume of the enzyme on Sephadex G-75).
2. A typical absorption spectrum with a maximum at 280 nm and a shoulder at 290 nm with no absorption in the visible region (consequently, no flavin or heme group).
3. The addition of electron acceptors did not affect the oxidation rate.
4. The pH optimum was 7.0.
5. Optimum temperature of 50°C, showing a plateau between 40 and 60°C. Most activity was lost at 80°C.
6. K_m under two sets of conditions were examined:
   6.1. (I) The reaction mixture contained 45 μmol of Tris-HCl (pH 7.2), 0.6 mg of bovine serum albumin and 0.07 μg of the enzyme;
   6.2. (II) The reaction mixture contained 40 μmol of Tris-HCl (pH 7.5) and 0.4 μg of the enzyme without bovine serum albumin. K_m values for both cases were 4.5 and 6.7×10^{-4} M, respectively.
7. The activity of the enzyme was seriously inhibited by the addition of $FeCl_3$, $CuSO_4$, $AgNO_3$, or $HgCl_2$. However, in common with *B. sterolicum* the addition of silver and mercury compounds was not inhibitory in the presence of either cysteine or glutathione.
8. Of the tested compounds that were capable of binding metals, only KCN or NaN_3 were capable of affecting enzymatic activity.

Details of the physical properties of *Streptomyces griseocarnus* are reported in a paper by Kernyi et al. [104].

A more recent study by Gadda et al. [105] compared the physical properties of COD from *S. hydroscopicus* (SCO) and from the recombinant enzyme from *B. ster* expressed in *E. coli* (BCO). Of the physical properties examined, only those relating to the prosthetic group were found to be markedly different. The common physical properties are listed below:

1. Both BCO and SCO were monomeric with an approximate mass of 55 kDa.
2. Acidic properties, with a pH between 4.4 and 5.1.
3. Similar spectroscopic properties for the prosthetic group with similar peaks at 370–390 and 440–470 nm.
4. A high pKa of approximately 11 for the N(3) position of the flavin.
5. Both form reversible flavin N(5)-sulphite adducts via discernable k_{on} and k_{off} steps.
6. The rearrangement of 5-cholesten-3-one to 4-cholesten-3-one is not rate limiting indicating that the rate-limiting step of the overall reaction is not the isomerisation.

Although both enzymes form reversible flavin N(5)-sulphite adducts, BCO has a higher affinity ($K_d \approx 0.14$ mM) compared with SCO ($K_d \approx 24$ mM). This data compared favourably with a midpoint redox potential which for BCO was about +100 mV greater than for SCO.

Nishiya et al. [68] has recently produced a more thermally stable form of the COD from a *Streptomyces* sp. The enhancement of thermal stability was achieved by means of in vitro random mutagenesis on a pre-targeted section of target DNA. The decision as to where the region of mutagenesis should occur was based on the previously recorded structure of choB determined by Vrielink et al. [99]. The region chosen was shown not to contain the FAD binding domain or active site ''hollow''.

The thermally stable mutants [68] were incubated at 50°C for a period of 16 h. COD activity was detected in four clones, although no activity was observed for the wild-type enzyme. The recombinant strains which still exhibited activity, and wild-type were cultured and purified to homogeneity, and the DNA sequence of the mutant section determined.

Each of the purified enzymes [68] migrated as a single protein band on SDS-PAGE. The molecular mass of the subunit was calculated to be ~55 kDa. The specific activities with 1.0 mM cholesterol, at 37°C and pH 7.0 of the mutants S103T, V121A, R135H and V145E were found to be 86.6, 72.0, 68.9 and 63.5 U/mg, respectively, while that of the wild type was 67.7 U/mg. The mutants were more thermally stable than the wild type at 50, 55 and 60°C. The half lives of the mutants were compared at 60°C and were 11.3 (S103T), 12.2 (V121A), 11.0 (R135H) and 24.1 min (V145E), while that of the wild type was 7.8 min.

Mutants [68] also exhibited similar pH stabilities in comparison to the wild-type. However, the pH optimum of one mutant (referred to as V145E) was shifted to pH 7.5 and also encompassed a broader range of acid and alkali.

The K_m values for cholesterol for S103T, R135H

and V145E were, 13, 30, and 11 μM, respectively, and compared to 13 μM for the wild type.

3.6. Pseudomonas sp.

Pseudomonas sp. [37] COD (CODP) has a molecular mass of ca. 56 kDa (estimated from gel filtration on a Sephadex G-150 column and SDS-PAGE). The purified enzyme was maximally active at pH 7.0 and 37°C, when activity was assayed in 100 mM potassium phosphate buffer. At pH values of 5 and 7, the activity was reduced to approximately one-third of the value of pH 7.0. There was no apparent loss at values of between 5 and 8, but a 25% loss occurred at pH 10. Mn^{2+} was the only metal ion observed to activate the enzyme, Fe^{2+}, Zn^{2+} and Hg^{2+} were marked inhibitors. At an incubation temperature of 70°C, full activity was observed for 5 min, and at 85% for a period of 30 min. A significant loss was reported at 80°C for 5 min incubation time.

The conversion of a Δ^5–Δ^4-ketosteroid has been shown to occur by Austin et al. [106] through an enolic intermediate. The mechanism for this conversion has been examined with 19-nortestosterone and D38N and Y14/D38N mutants by UVRR (ultra-violet resonant Raman). The frequencies of UVRR bands associated with C═O and C═C stretching was used to monitor the enone portion of the steroid and the effects of side chains. The mutants produced lower polarising effects and suggested that most of the polarisation of the conjugated ketone can be attributed to hydrogen bond donation by the hydroxyl group of Tyr-14. A smaller contribution of Asp-38 was detected, which in part, was cooperative with that of Tyr-14 [106].

On the basis of the bacterial species identification by Hawkinson and Pollack [107], the strength of the 19-nortestosterone polarisation produced by the native enzyme is intermediate between complete protonation and the hydrogen-bonding environment of 10 M hydrochloric acid [106]. Since the UVRR (ultraviolet Resonance Raman) spectrum of Tyr-14 is unperturbed upon binding of the steroid, the hydrogen bond to the carbonyl group of 19-nortestosterone may be compensated by a second hydrogen bond to Tyr-14 from another donor, possibly a backbone NH or a bound water molecule. ''This compensating hydrogen bond could lower the free energy of the enzyme transition state, in which the hydroxyl proton of Tyr-14 helps to dissipate the negative charge that accumulates on the steroid carbonyl group [106]''.

However, it should be made clear that the rate of cholesterol oxidation via COD in homogenous water–organic mixtures without surfactants has been seen to vary not only according to the nature of the organic solvents and pH (in agreement with most publications findings), but also with buffer salts and the ''age'' of the cholesterol solutions [108]. Solutions prepared 24 h prior to the reaction exhibit large reductions in maximal rate, the K_m with cholesterol and the effective molar extinction co-efficient for 4-cholesten-3-one. Nevertheless, upon sonication it was observed that these parameters compared to values obtained with freshly prepared solutions.

3.7. Novel products of COD action upon cholesterol

It has been reported that certain strains of COD produce unusual products upon reaction with cholesterol. Due to the possibility of cholesterol autoxidation giving rise to (non-enzymic) oxygenation at the 6 and 7 positions on the steroid nucleus, it is possible that the results described by the two groups of workers [62,109] require further investigation. Molnar et al. [62] found that the following four strains produced a 4-cholesten-6-ol-3-one from prolonged reaction with cholesterol and 4-cholesten-3-one:

1. Recombinant strains of *Streptomyces lividans* and *E. coli* carrying cloned genetic fragments of *Streptomyces* sp. SA-COO;
2. *Brevibacterium sterolicum*;
3. A *Pseudomonas* sp. and;
4. A highly recombinant *Streptomyces*.

A concurrent branched reaction pathway was predicted for the production of the two metabolites.

Teng and Smith [109] have shown that commercially available *Pseudomonas fluorescens* cholesterol oxidase not only converted cholesterol to the expected product, 4-cholesten-3-one, but also catalysed the transformation to 6β-hydroperoxycholest-4-*en*-3-one as the initial product, without the production of 4-cholesten-3-one. The transformation indicates that *P. fluorescens* cholesterol oxidase also acts as a flavoprotein dioxygenase.

3.8. Enzymatic reaction in a water-in-oil microemulsion

Reactions of cholesterol oxidase in organic media or solvent/aqueous microemulsions have become the subject of a great deal of interest in recent years. The advantage of such systems lies in their ability to dissolve water insoluble analytes. Until the mid-eighties, nearly all enzymatic analyses were performed in aqueous media since water was thought an essential requirement for activation. However, this conclusion has proven to be unfounded since various enzymes have been shown to be active in nearly anhydrous solvents [110–114] of various biphasic and ternary systems. Microemulsions are uniquely suited media for the COD enzy-

mic reaction, as they provide an enormous interfacial area in which reactants can interact with the entrapped enzyme [115]. Hedstrom et al. [116] examined the role of water in maintaining a water/oil microemulsion consisting of sodium *bis*(2-ethylhexyl) sulfosuccinate/isooctane/water and related their findings to the reaction characteristics of COD (*B. ster*) in the reverse micelles. Backland and Rantala [117] examined the catalysis in various water–alkane–soybean lecithin microemulsion systems (Winsor I → Winsor IV). A Winsor IV system afforded the highest yield at room temperature, without the addition of extra enzymes and within a reasonable reaction time. Slotte and Gronberg [118] has also examined the oxidation of cholesterol in native low and high density lipoproteins and in monolayers prepared from the surface lipids. Their results showed that the surface pressure of native LDL and HDL_3 was less than 25 and 20 mN/m, respectively. Further, it was observed that LDL cholesterol was oxidised at a much lower rate than HDL_3.

4. Substrate specificity

4.1. General

Substrate specificity experiments have been detailed for most of the commercially available sources and include *Nocardia* [119–123], *Brevibacterium* [124–127], *Streptomyces* [103] and *Corynebacterium* [28]. The rate of oxidation with respect to cholesterol has been compared for *Nocardia*, *Brevibacterium*, *Schizophyllum* and *Streptomyces* [128]. The substrate specificities of *Nocardia* and *Brevibacterium* have been discussed at length in the review by Smith and Brooks [13]. The enzymatic reaction consists of an oxidation step at the C_3 position, followed in the case of 5-*ene*-steroids by isomerisation of the C_5–C_6 position. Some investigations have involved estimating the relative rates of conjugated enone formation by the increase in UV absorption. However, this is a measure of both steps, and, moreover, cannot be applied to the oxidation of 5α-3β-hydroxysteroids. The rate of the oxidation step can be measured by monitoring oxygen uptake or hydrogen peroxide production, and both methods have been applied to the enzymes from *Nocardia* sp. and *B. sterolicum*.

4.2. Rhodococcus sp.

In 1992 and 1994, respectively, Kreit et al. [45,50] examined the substrate specificity of COD from the source *Rhodococcus* sp. GK1, a soil-isolated strain with several sterols. Of those examined (the names of the specific sterols used in this study used were not provided), the enzyme was found to be most active with cholesterol (100%), β-sitosterol (70%) and stigmasterol (40%). Sterols with modified A-rings and B-

Table 1
Apparent K_m and relative V_{max} values for the oxidation of some 3-hydroxysteroids by cholesterol oxidase from *N. erythropolis* [13][a]

3-Hydroxysteroid	Km (μM)	Rel V_{max}
Cholesterol	2.9	100
5α-Cholestan-3β-ol	2.3	78
4-Cholesten-3β-ol	3.4	111
5α-cholest-7-en-3β-ol	10.7	64
5α-Choles-8(14)-en-3β-ol	2.8	17
5,7-Cholestadien-3β-ol	5.5	32
4α-Methylcholesterol	12.5	0.2
4β-Methylcholesterol	28.4	1.8
4,4-Dimethylcholesterol	7.5	0.15
24-Ketocholesterol	2.7	132
Sitosterol	6.6	105
Stigmasterol	5.9	34
Fucosterol	11.5	88
Diosgenin	13.9	3.2
20-Hydroxycholesterol (20*S*)	4.2	104
22-Hydroxycholesterol (22*RS*)	5.1	90
20,22-Dihydroxycholesterol (20*R*, 22*R*)	7.1	93
24-Hydroxycholesterol (24*RS*)	4.3	129
25-Hydroxycholesterol	1.5	61
26-Hydroxycholesterol	1.2	56
25-Hydroxy-27-norcholesterol (25*RS*)	1.4	63
5-Cholene-3β,24-diol	4.8	85
23,24-Dinor-5-cholene-3β,22- diol	9.3	73
5-Pregnene-3β,20β-diol	3.7	22
17β-(Hydroxymethyl)-5-androsten-3β-ol	3.1	1.7
5-Androstene-3β,17β-diol	1.2	0.6
5-Androstene-3β,17β-diol 17-benzoate	5.4	1.7
Dehydroepiandrosterone (DHA)	3.3	0.9
DHA 17-oxime	4.9	0.5
DHA 17-*O*-methyloxime	1.8	0.8
DHA 17-*O*-ethyloxime	1.9	1.5
DHA 17-*O*-*sec*.-butyloxime	23.8	3.8
DHA 17-*O*-isopentyloxime	27.1	31
DHA 17-*O*-benzyloxime	22.5	59
20,25-Diazacholesterol	124	7.8
5β-Cholestan-3β-ol		_[b]
5-Cholesten-3α-ol		_[b]
4-Cholesten-3α-ol		_[b]
5β-Cholestan-3α-ol		_[c]
Cholecalciferol		_[c]
Solasidine		_[b]
Solanidine		_[b]

[a] Steroids dissolved in propan-2-ol (50 μl) were mixed with 2.75 ml of 50 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) containing 1 mg of Triton X-100/ml, 0.1 ml of 4-aminoantipyrine (2.4 mM) and 0.1 ml of phenol (0.4 M) at 30°C. In most instances, the steroids were oxidised with 0.02 U of cholesterol oxidase. The hydrogen peroxide produced was monitored using horseradish peroxidase (0.18 U) which oxidatively coupled the 4-aminoantipyrine and phenol to give a quinoneimine absorbing at 500 nm. Provisional values of K_m and V_{max} were obtained by the graphical method of Eisenthal and Cornish-Bowden [133].

[b] Detectable rate but too low for kinetic measurements.

[c] No detectable oxidation.

rings, or the 3α-OH of cholic acid remained unoxidised.

4.3. Cholesterol oxidase from Nocardia sp.

Nocardia sp. enzymes are particularly influenced by the side chain, as originally found by Richmond [3]. Long side chains appear to aid the orientation of the substrate with respect to the active enzymatic site. Allain et al. [129] found that ergosterol, 5,7-cholestadien-3β-ol, 20α-hydroxycholesterol, 5α-cholestan-3β-ol and 7-cholesten-3β-ol were oxidised at rates lower than that of the rate of cholesterol. In these experiments, the kinetics of oxidation were followed since it was the rate of H_2O_2 produced that was measured. Wortberg [121] observed no oxidation of a natural mixture of 4-methylsterols, or of lanosterol (the effects of 4-methyl groups are illustrated in Table 1). Two points are of particular significance from these three workers. Although increasing the C-17 side chain lengths (from no side chain up to that of cholesterol) was shown to enhance oxidation, the maximum activity was still observed with cholesterol. Smith and Brooks [13] also examined the double-bond isomers of cholesterol, 4-cholesten-3β-ol, 5α-cholest-7-*en*-3β-ol and 5α-cholest-8(14)-*en*-3β-ol. The variations in K_m and relative V_{max} values were attributed to a shift in the binding of the 3β-hydroxyl group relative to the active site. The following is a more detailed discussion.

Richmond [3] originally examined the substrate specificity of cholesterol oxidase from *Nocardia* sp. NCIB 10554 by measuring the rate of increase in extinction due to the formation of the conjugated enone system relative to the rate with cholesterol. 4-Cholesten-3β-ol was oxidised more slowly (77%). Data for a small group of 3β-hydroxy-5-*enes* gave some indication of the importance of the C-17 side chain: relative oxidation rates were: 3β-hydroxy-5-cholen-24-oic acid, 23%; 5-pregnen-3β-ol, 6.3%; 5-androsten-3β-ol, 0.9%. 5α-Cholestan-3β-ol and 5α-cholestan-3β-ol were recorded as remaining unoxidised: however, the UV assay system would not have revealed the formation of a saturated 3-ketone. A K_m for cholesterol of 1.4×10^{-5} M was determined.

Using the same enzyme source, Allain et al. [129] found that ergosterol, 5,7-cholestadien-3β-ol, 20α-hydroxycholesterol, 5α-cholestan-3β-ol and 7-cholesten-3β-ol were oxidised at rates in the range 60–82% of the rate for cholesterol. In these experiments, the hydrogen peroxide generated was measured by the oxidative coupling of 4-aminoantipyrine with phenol by horseradish peroxidase to give a quinone-imine absorbing at 500 nm [130]. The COD from this source has been explored for the mechanism of both, its oxidation and isomerisation by several workers. The substrate specificity of the oxidase action has been examined in detail [119–122]. The presence of only a C1 side chain on the rate of isomerization action by using cholest-5-*en*-3-one, pregn-5-*ene*–3,20-dione and androst-5-*ene*-3,17-dione have not indicated any major influence of the C-17 side chain on the rate of isomerization; however, in the absence of a side chain, a large increase in the apparent K_m was observed. Smith and Brooks [131] also examined the mechanism of isomerization of cholest-5-*en*-3-one to cholest-4-*en*-3-one by incubations with [4α-^{2}H]cholesterol and [4β-^{2}H]cholesterol. Incubations in ^2H_2O buffer were also performed to detect incorporation of ^{2}H from the medium. On the basis of IR evidence, an intramolecular *cis*-diaxial transfer of hydrogen during isomerisation was envisaged, as was described for the COD from *Pseudomonas testeroni* [132]. However, the latter enzyme may not achieve isomerization in such a stereospecific manner, since it was noted that there was some loss of ^{2}H from the [4α-^{2}H]cholesterol and there was evidence that deuterium may have been transferred to the 6β-position. MS and IR evidence additionally showed ^{2}H incorporation at the 6β-position from the incubation medium.

The cholesterol oxidase isolated from *N. erythropolis*, as supplied by Boehringer-Mannheim, has been the subject of two independent studies of substrate specificity. Wortberg [121] has demonstrated the rate of oxidation of a number of 3-hydroxysteroids. In this case, the hydrogen peroxide formed was monitored using peroxidase but with 2,2′-azino-*di*-[3-ethyl-benzthiazoline sulphonate] as a chromogen. The initial rates of oxidation were compared with that for cholesterol (Table 1). 4,24-Cholestadien-3β-ol, 5α-cholestan-3β-ol, 4-cholesten-3β-ol, campesterol, sitosterol and stigmasterol were oxidised more slowly than cholesterol. The lowest measured rate was observed with ergosterol, and when this is compared to 7,*E*-22-ergostadien-3β-ol it is evident that the C_5 bond when in conjugation is less easily oxidised. 5-cholesten-3α-ol and lanosterol did not serve as substrates.

In initial studies by Smith and Brooks [119], relative rates of ketone formation were measured by UV spectroscopy or by GLC: more detailed work was later based on measurements of hydrogen peroxide production using the peroxidase-chromogen system described by Allain et al. [129]. Sterols were solubilised by addition of isopropanol solutions to the phosphate buffer containing Triton X-100. Kinetic constants (K_m and V_{max}) were determined and are summarised in Table 1. Salient features of the results are discussed below.

The double bond isomers of cholesterol, 4-cholesten-3β-ol, 5α-cholest-7-*en*-3β-ol and 5α-cholest-8(14)-*en*-3β-

ol, together with 5,7-cholestadien-3β-ol were compared. The variations in K_m and relative V_{max} values (Table 1) were ascribed to the distortion of the ring system, causing either a change in the degree of binding to the enzyme or a shift in the position of the 3β-hydroxy group relative to the active site. The effect of the side chain on the reactivity of various 3β-hydroxy steroids is also illustrated in Table 1 [13]. These steroids range from dehydroepiandrosterone (a poor substrate), to some oximes at the C_{17} position to simulate longer side chains.

4.4. Brevibacterium sterolicum

In contrast to the enzyme from *Nocardia* sp., the COD from *B. ster* is fairly reactive towards substrates without a side chain. Uwajima et al. [124] incubated this enzyme with a number of steroids, and the relative enzymic oxidation rates were calculated by determining oxygen uptake manometrically: typical comparable results were: cholesterol (100), dehydroepiandrosterone (41), pregnenolene (22), sitosterol (20), 5α-cholestan-3β-ol (13), and stigmasterol (10). Ergosterol, diosgenin, cholecalciferol, cholic acid, digitoxigenin, 5α-androstane-3α,17β-diol, androsterone, testosterone and oestradiol were unreactive. Yamaguchi et al. [125] reported that dehydroepiandrosterone and epiandrosterone are substrates of the enzyme from *B. ster* in a reaction medium that was analogous to that of Allain et al. [129]. Androsterone, etiocholanone, pregnanediol, testosterone (which already is a 4-*en*-3-one), estrone, estriol and cortisol were reported to be non-substrates by Yamaguchi et al. [125].

Ikawa et al. [127] found that *B. ster* COD was capable of oxidising sterols, steroid hormones, and bile acids having a free 3β-hydroxyl group. Variations in the length of the side chain had no marked effect on activity. Relative reactivities were found as follows: Cholesterol (100), 7α-hydroxycholesterol (98.7), 7β-hydroxycholesterol (82.3), 7-ketocholesterol (60.6), desmosterol (85.4), ergosterol (51.0), β-sitosterol (65.2), zymosterol (76.2). Cholesteryl acetate, cholesteryl benzoate, cholesteryl palmitate, cholesteryl stearate, lanosterol, dihydrolanosterol, 4-cholesten-3- one and 5-cholesten-3α,7α-diol were noted as being unreactive. Of the steroid hormones, dehydroepiandrosterone reacted with an activity similar to cholesterol. Epiandrosterone (97.3), pregnenolone (90.2) and 5α-pregnan-3β-ol-20-one (9.7) were also relatively reactive. Typical results with bile acids were: 3β-hydroxycholanoic (1.5), 3β-hydroxyallocholanoic (1.4), 3β-hydroxychol-4-*enoic* (98.2), 3β-hydroxychol-5-enoic (100.0), 3β,6β-dihydroxyallocholanoic (2.1), 3β,7α-dihydroxycholanoic (1.8), 3β,7α-dihydroxychol-5-enoic (100), 3β,7β-dihydroxychol-5-enoic (56.8), 3β-hydroxy-7-oxochol-5-enoic (75.9).

In 1994, the COD from *B. sterolicum* was modified by hydrogen peroxide by Loubat-Hugel et al. [78]. The modified enzyme is unusual in being inactive with cholesterol solubilized in buffer with surfactants. However, pregn-5-*en*-3β-ol, 3β-hydroxy-androst-5-*en*-17-one and 3β-hydroxy-androst-5-*en*-17β-carboxylic acid when solubilized in the same conditions were found to be substrates of the modified enzyme. Furthermore, the modified enzyme showed 60 and 22% residual activities towards cholesterol solubilised, respectively, in a microemulsion and in a biphasic system. The observed loss of activity for cholesterol was attributed to two possibilities. The oxidized cholesterol oxidase was no longer capable of extracting cholesterol from mixed cholesterol/surfactant aggregates or was unable to recognise steroids with a shorter side chain. Since cholesterol remained a substrate solubilised in a microemulsion/biphasic system, the residual activity was thought likely to be due to a conformational change in these media.

4.5. Streptomyces sp.

4.5.1. General

Tomioka et al. [103] examined the substrate specificity for *Streptomyces violascens* quite extensively in 1976. The relative rates of oxidation were followed by the measurement of hydrogen peroxide formation. The enzyme attacked most of the 3β-hydroxy-steroids the authors examined, with the exceptions of 5β-cholestan-3β-ol and 5α-lanosta-8,24-dien-3β-ol. Relative rates for example with respect to cholesterol were: 5α-cholestan-3β-ol (91), 5α-cholestane-3β,5α-diol (88), androst-5-*en*-3β-ol-17-one (80), pregn-5-*en*-3β-ol-20-one (83) and 5α-pregnane-3β,16β,20α-triol [23]. The substrate specifity of *S. violascens* appears to be generally similar to *B. ster* but differs markedly from *Nocardia* sp.

4.5.2. Orientated monolayer substrate specificity

A particular problem of the early substrate specificity studies was the inability to control the orientation of the substrate relative to the enzyme. Slotte's group examined the oxidation of cholesterol in monolayers and showed that cholesterol present in a monolayer is a substrate for the enzymes from *Streptomyces* and *Brevibacterium* sp. [98]. In mixed monolayers containing phospholipids, acyl chain and head group composition have also been shown to affect the rate of COD activity [118,134–138]. COD from *Streptomyces cinnamomeus* was examined in the rigid confines (Langmuir type experiment) of oriented sterol monolayers at an air/water interface [21]. The rates of oxidation were measured as a backward movement of the barrier due to an oxidation-dependent increase in the monolayer area. Of the cholesterol analogues with alterations in ring structure that were examined (e.g. 5-

cholesten-3β-ol, 5α-cholestan-3β-ol, 4-cholesten-3β-ol, 5α-cholest-7-*en*-3β-ol, 5,7-cholestadien-3β-ol, 3β-hydroxy-5-cholesten-7-one and 5β-cholestan-3β-ol) it was observed that 5α-cholestan-3β-ol was oxidised almost as fast as cholesterol itself (which compares well with corresponding results from Tomioka [103]). When the Δ^5-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 oxidised 5.1-fold slower compared to cholesterol, whereas (as expected), 3β-hydroxy-5-cholesten-7-one and 5β-cholestan-3β-ol were not substrates of the enzyme (differing results from those of Tomioka [103]). C-17 side chain analogues of cholesterol (compounds studied included 5-androsten-3β-ol, 5,24-cholestadien-3β-ol and 24β-ethyl-5,22-cholestadien-3β-ol), 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 (24β-ethyl-5,22-cholestadien-3β-ol) had no appreciable effects on sterol oxidation rate, implying that the enzyme did not recognise the side chain in oriented sterol monolayers [21].

However, a separate study by Slotte et al. [138] examined the rate of oxidation by COD in small unilamellar vesicles containing either 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine or 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. The rate of oxidation increased as a function of increasingly branched C-17 side-chain length. A similar trend was observed for the unbranched sterol series studied.

4.6. Pseudomonas sp.

Relatively little is known of the substrate specificity of *Pseudomonas* sp. Lee et al. [87] has examined the substrate specificity as shown in Table 2.

The measurement of relative oxidation rates of various steroids by the enzyme were measured by the formation of hydrogen peroxide. In common with other sources of COD enzymes, the 3β-hydroxy configuration was essential for activity. The enzyme did show some differences in specificities in comparison with other sources. The enzyme catalysed the oxidation of dihydrocholesterol at 78% of the rate for cholesterol which is also the preferred substrate of *Streptomyces violascens* [103] and *Streptoverticillium cholesterolicum* [17]. In common with *Nocardia* sp. [119,120], this enzyme also appears to be particularly influenced by the C-17 side chain, since dehydroisoandrosterone was not a substrate. In contrast, *Streptomyces violascens* [103] and *Streptoverticillium cholesterolicum* [17] are active with this steroid. The results indicate further that a 3β-hydroxyl and the presence of a side chain with more than two carbon atoms at the C17 position are in most cases essential for substrate oxidation.

Table 2
Substrate specificity of *Pseudomonas* sp. strain COX629

Substrate (0.5 mM)	Relative activity (%)
Cholesterol	100
Dihydrocholesterol	78
Ergosterol	51
Stigmasterol	40
Stigmastanol	32
7-Dehydrocholesterol	24
Testosterone	0
5-Cholestene	0
Dehydroisoandrosterone	0
Cholesteryl linoleate	0

4.7. Inhibitors of COD action

Morpholine derivatives, especially fenpropimorph and tridemorph, have been shown to block selectively the isomerisation activity of cholesterol oxidases isolated from *Nocardia erythropolis*, *Streptomyces* sp., *Pseudomonas testeroni* and *Schizophyllum commune* [139]. These enzymes show many differences in physical characteristics and catalytic behaviour. The effectiveness of the inhibitors varied with the cholesterol oxidase tested. Fenpropimorph was most effective with each of the four enzymes, 50 mg/l inhibiting about 50% of the enzyme activity. Inhibition was instantaneous and followed a reversible competitive mechanism in *Streptomyces* sp. and a reversible non-competitive mechanism in *Nocardia erythropolis* and *Schizophyllum commune*. An irreversible type of inhibition was observed for *P. testeroni* cholesterol oxidase.

5. Analytical applications

5.1. General

In the following section, the work has been reviewed under the categories: plasma/serum/lipoprotein fractions and cellular analysis.

The use of cholesterol oxidase as an analytical probe has been extended to several novel applications such as the following examples below:

(a) The (c.f. Section 5.2) determination of cholesterol in various sample types are exemplified below:
   (i) total and esterified serum [3,1,129,92,140–142];
   (ii) low density to high density lipoproteins (LDL-HDL): LDL [143]; HDL, [144–148].

(b) The determination of cholesterol (c.f. Section 5.3) on the cell membrane of erythrocytes, other cells and cellular compartments, and to test the cell membrane disruption caused by the enzyme [149–163].
(c) The determination of total cholesterol in gall stones [164], and in human bile [165,166].
(d) The determination by chemiluminescence methods [167,168], and also by fluorescence [142] methods.
(e) The enzyme has also been incorporated into several biosensors (electrochemical), either in its free form or after some form of modification into an inert polymer matrix [169–176].

In general, most methods rely on the coupling of H_2O_2 to indicate the relative concentration of cholesterol indirectly. There are several reasons for this. Firstly, coupling of hydrogen peroxide with chromophoric compounds yields adducts that display highly absorbing or highly fluorescent chromophores which permit much more sensitive measurements of cholesterol than by the measurement of the Δ^4-3-ketone chromophore would allow. Secondly, the redox couple of H_2O_2 is clearly identified which allows a facile voltammetric and amperometric measurement. Thirdly, interfering compounds in plasma may require the formed Δ^4-3-ketone to be extracted before measurement can be made. Some exceptions to these methodologies are discussed, in which direct measurements of Δ^4-3-ketones are made.

5.2. Plasma/serum/lipoprotein fractions

The determination of serum cholesterol is very important for the assessment of atherosclerosis and other lipid disorders and for the estimation of the risk of thrombosis and myocardial infarction. Prior to enzymatic determination, chemical analyses based on the Liebermann-Burchard reactions were prone to interference from other serum constituents (haemoglobin, bilirubin etc.) and required the use of corrosive reagents. By 1952, most of the problems of the interferences in the Liebermann-Burchard methodologies had been solved by Abell et al. [177] and, notwithstanding the problems caused by corrosive reagents, this method can still be used as an alternative to the use of modern cholesterol oxidase methods in quality control studies. In 1972, Richmond [3] demonstrated the use of cholesterol oxidase from Nocardia for the measurement of serum cholesterol, by the assay of the hydrogen peroxide produced; but relied on the non-enzymic hydrolysis of the cholesterol esters. Flegg [1] in 1973 used COD (*Nocardia*) for the assay of serum cholesterol but it required the lengthy incubation time of 2 h and the extraction of the product 4-cholesten-3-one to be detected by direct UV measurement at 240 nm. In Richmond's method [3], the hydrogen peroxide produced during the oxidation, reacted with quadrivalent titanium and xylenol orange to give a complex absorbing at 550 nm. By 1974, Allain et al. [129] introduced the use of the cholesterol ester hydrolase (EC 3.1.1.13) to dispense with the need for an alkaline saponification step; which allowed the determination of total cholesterol in serum (including the esters) by a totally enzymic method. The subsequent production of H_2O_2 by the oxidase is measured with horseradish peroxidase to effect the oxidative coupling of 4-aminopyrine with phenol (CHOD-PAP), resulting in a quinone-imine absorbing at 500 nm [129]. The longevity of the method is due to the distinct advantages of simplicity, little interference from urea, ascorbic acid, creatinine, glucose, bilirubin, uric acid, haemoglobin or sodium bromide and is additionally highly reproducible. This method is still routinely used in clinical laboratories; and has been compared with the Reflotron dry chemistry method (Boehringer-Mannheim, Germany) by Lapinleimu et al. [141] (1994) in a study of 4150 venous blood samples. The results have shown that the CHOD-PAP method was more sensitive but slower than the Reflotron (requiring only a "pin-prick" of blood from the finger) method.

The HPLC-UV determination of the cholesterol product 4-cholesten-3-one (240 nm) from rat serum and thoracic duct lymph chylomicrons (sub-nanomolar) has been described by Murata and Ide [140], and illustrates that the horseradish peroxidase coupling reaction need not be used in some cases to yield a better detection limit (0.15 nmol) than conventional methods. Unsaponifiable lipids from rat serum and thoracic duct lymph chylomicron samples were treated with cholesterol oxidase. The resulting 4-cholesten-3-one was analysed via HPLC with hexane/isopropanol (95:5, v/v) as mobile phase and detected with a UV spectrometer at 240 nm. Excellent correlation of peak areas for a given amount of authentic cholesterol was obtained. The detection limit (0.09 nmol) obtained from reference cholesterol mixtures and 0.15 nmol for a sample preparation compares favourably with the 30 nmol determined by conventional methods.

Brooks et al. [92] have applied selective modification of traces of polar minor sterols from human serum by COD from *B. ster* in order to identify and quantify these moieties by capillary GLC. The resulting Δ^4-3-one derivatives have characteristic retention times and mass spectra which aids in their identification typically in a complex mixture of sterols and other interfering compounds. Furthermore, the Δ^4-3-ketones are useful for the detection and recovery of sterols from a TLC plate and could be of use in combinatorial organic synthesis and subsequent drug screening (chemical neighbourhoods) studies due to the selective nature of the enzyme cavity.

Sasamoto et al. [168] used the following method with chemiluminescent detection of the reaction between lucigenin and H_2O_2. Buffered serum containing cholesterol oxidase and esterase and were introduced followed by an alkaline solution of lucigenin. The chemiluminescent intensity was measured after five seconds with a 10 s incubation period. The detection limit was 1 mg/l and the RSD (relative standard deviation) for the determination of 141–267 mg/l cholesterol in serum was 2.3–4.2%. Mike et al. [167] also used a temperature enhanced chemiluminescent flow through reaction system with a pre-mixed reactant solution of methanolic triethylamine and *bis*-(2,4,6-trichlorophenyl) oxalate-perylene in ethyl acetate for reaction with H_2O_2. The temperature of the system was optimised leading to an enhancement of the signal-to-noise ratio. The detection limit thus obtained was 7.2 mg/l in serum.

Gray [142] used 4-hydroxyphenylacetic acid in the presence of peroxidase for a fluorimetric determination at 320 nm (excitation at 400 nm). The detection limit was 0.5 nmol/ml (in serum) and the results correlated well ($r = 0.9$) with those by a colorimetric method and isotope dilution MS.

Polyethylene glycol-modified COD has been shown to permit the direct measurement of high density lipoprotein cholesterol in serum containing added α-cyclodextran sulphate and a small amount of dextran sulphate without precipitation of lipoprotein micelles. This is largely due to the selectivity of modified enzyme alone, however in the presence of magnesium ions, the α-cyclodextran sulphate reduced the reactivity of chylomicrons and VLDL cholesterol towards the enzyme. The results obtained for serum HDL assay correlated well with established techniques such as precipitation-based and ultracentifugation methods.

Sugiuchi et al. [147] used a similar method to that of Allain et al. [129] for the assay of HDL cholesterol but used PEG modified COD and esterase. Sulphated α-cyclodextran was used for precipitation and the results compared favourably with those obtained by a dextran sulphate-phosphotungstate/$MgCl_2$ method. By 1998, Sugiuchi et al. [143] had developed an automated method for the measurement of serum LDL cholesterol without ultracentifugation separation; using a nonionic surfactant, polyoxyethylene-polyoxypropylene block copolyether (POE-POP), magnesium ions, and a sodium salt of sulfated cyclic maltohexaose, α-cyclodextrin sulfate. They reported a good correlation between the results of LDL-cholesterol assayed by the proposed method and the beta-quantification reference method involving 161 sera with triglyceride concentrations ranging from 0.3 to 22.6 mmol/l.

Moshides [148] described an HDL-cholesterol measurement procedure involving enzymatic oxidation incorporating 2,4,6-tribromo-3-hydroxybenzoic acid instead of phenol on a Cobas Bio centrifugal analyser. The different precipitation methods for HDL and the effects of ascorbic acid intereference have also been studied. The substitution of phenol by 2,4,6-tribromo-3-hydroxybenzoic acid resulted in a higher absorption coefficient. The method has been devised for the sequential measurement of serum triglycerides and total cholesterol.

A rapid bioreactor method has been devised by Makino et al. [169] for the sequential measurement of serum triglycerides and total cholesterol. Classes of lipoproteins in serum have been separated by size-exclusion chromatography the eluate being passed over G6000PW gel on which cholesterol esterase and oxidase have been co-immobilised (preparation described in original paper). The eluate was combined with a solution of Triton X-100, 4-aminoantipyrine, *N*-ethyl-*N*-(2-hydroxysulphopropyl)*m*-toluidine and HRP (horseradish peroxidase) before introduction into a heated reaction vessel/absorbance chamber for detection at 550 nm. The detection limit in the rection vessel was 2 μg of cholesterol.

The primary drawback of colorimetric techniques based upon the detection of quinoneimine dye is the necessity to remove optical interferences caused by contaminants such as haemoglobin in blood. A more convenient determination can be made using O_2/H_2O_2 as the electron mediator for COD. Recently, however Nakaminami et al. [175] examined several novel mediators for their ability to accept electrons from COD. Of those tested 1-methoxy-5-methylphenazinium was found to be the most effective. Although the electrochemical detection of cholesterol was achieved using dissolved COD and the mediator thionin, the response current was highly influenced by the presence of dissolved oxygen. In addition, the system required more than 10 min to obtain a steady current response. Electro-oxidisable compounds such as uric acid, ascorbic acid, and 4-acetamidophenol gave no significant oxidation current at 0 V versus SCE where thionin was significantly oxidised. The difference between the absence and presence of interfering compounds only influenced the i_r values by 2.6%.

Oyama et al. [171] prepared a cholesterol sensor based on the electrochemical reduction of oxygen. The sensor was based on a bilayer-film coating prepared by the method used for glucose sensors. The basal-plane pyrolytic graphite electrode substrata were coated first with a redox active Co-2,2′,2″,2″-(21H, 23H-porphine-tetraethyl)tetra-aniline polymer by electropolymerisation and then with an enzyme film consisting of bovine serum albumin and cholesterol oxidase. The sensor was designed to be subject to less organic interferences because the sensor was based on oxygen measurement. Furthermore, the sensor was simple to fabricate and gave reproducible behaviour.

In Cheillan's procudure [172], the cholesterol oxidase was immobilised on nylon nets placed over the gas membrane of an oxygen probe (Oxyliquid, Model 233, Idronaut, Italy) or over the cellulose acetate membrane of an H_2O_2 detector (Yellow Spring, USA). Both assemblies when covered with a polycarbonate membrane allowed extension of the linear range. When incorporated in a flow injection system the H_2O_2 device provided the fastest and most reproducible detection. The sensor was linear over 0.5–5 mM-cholesterol, or up to 10 mM when the polycarbonate membrane was fitted.

Trettnak and Wolfbeis [170] built a fibre-optic biosensor consisting of COD immobilised with 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide on a Biodyne C nylon membrane and fixed to an O-sensitive layer of butylated decacyclene in silicone polymer with a black O-permeable PTFE membrane sandwiched between them. Light was guided through a bifurcated optical fibre bundled to the through cell and the fluorescence was guided back to the photometer through the output channel. The cholesterol solution to be analysed was prepared in isopropanol, mixed with Triton X-100, and then diluted with sodium phosphate buffer of pH 7.9 to give a final pH of 7.25. The analytical range was 0.2–3 mM and the time taken to reach a steady state in a flowing solution was 7–12 min.

5.3. Erythrocytes, other cells and cellular compartments

Although COD is primarily used for the analysis of serum/lipoprotein cholesterol analysis, it has also been applied to probe cellular cholesterol levels and/or total sterol levels. In the cases where whole cells are incubated, only membrane cholesterol and other minor sterols are amenable to oxidation, since incubated cells are not lysed with incubation with the enzyme. Total assay of the cell (usually, but not always) requires the sterols to be first isolated by solvent extraction and then modification with COD. This permits the analysis of 3β-hydroxy-sterols via oxidation to the Δ^4-3-ones chromophoric group, which then facilitates in the identification of the structurally unknown sterol.

Cholesterol and related sterols for example have been assayed in sickle-cell and healthy erythrocytes by Teng and Smith [162]. Sterols were extracted from cleaned (rinsed with PBS) cells with $CHCl_3$/MeOH (2:1 v/v). The solvent was removed and replaced by propan-2-ol for oxidation (1 h) with COD. The COD was added as a solution comprising Tris buffer of pH 7.8 at 37°C in a medium of PBS and sodium cholate and Tween 20 in phosphate buffer of pH 7.5. After this period, methanol was added to terminate the reaction, and the products were extracted into light petroleum and transferred into $CHCl_3$ for HPLC on a Ultrasphere SIL (5 μm) with use of a Perisorb A precolumn, with hexane/propan-2-ol (50:1) as mobile phase (1 ml/min) and detection at 210 nm (oxysterols) and 235 nm (ketones). The relative retention times for the 11 oxysterols and 10 ketones identified were evaluated and tabulated as reference data to aid in the identification of blood samples. This was also carried out for the same oxysterols and ketones on fluorescent coated silica TLC plate with an elution solvent of ether/hexane (3:2). The method is similar to the one adopted by Goh et al. [155,156] for the measurement of desmosterol, 7-dehydrocholesterol, and cholesterol which was, however, accomplished without the use of organic solvents.

Contreras et al. [159] used HPLC in the analysis of cholesterol in cultured cells. In this case the cholesteryl esters were hydrolysed quantitatively with incubation of cholesterol esterase. The total cholesterol was then converted to 4-cholesten-3-one for UV detection at 240 nm. The method has the advantage that cell components did not interfere and prior lipid extraction was found to be unnecessary. Additionally, the sensitivity was low enough to permit the determination of free and total cholesterol in as few as 5×10^3 cells.

A fluorimetric assay has been developed by Petrack and Latario [160] for the measurement of the hydrolytic activity of cholesterol esterase in rat tissues and the synthesis of 27-hydroxycholesterol in rat mitochondria. Mitochondria were prepared from rat livers and were incubated at 37°C for 15 min in a solution containing phosphate (pH 7.5), dithiothreitol, EDTA, NADPH, trisodium citrate and isocitrate dehydrogenase. The reaction was stopped by the addition of 40% sodium cholate solution. Blank mixtures were prepared by the addition of sodium cholate before the mitochondria. Cholesterol oxidase was then added and the mixture was again incubated at 37°C for 20 min to generate the α,β-unsaturated ketones. The reaction was then stopped by the addition of methanol. The internal standard (testosterone propionate) was added and the mixture was extracted with hexane. The organic layer was evaporated to dryness under nitrogen and the residue was redissolved in 5% propan-2-ol solution in dodecane. An aliquot was removed and analysed on a Spherisorb silica column (25 × 4.6 mm) with hexane/propan-2-ol (19:1) as mobile phase (1 ml/min) with detection at 240 nm.

Other examples of cholesterol analyses in cells are briefly described below. In various papers (published between 1984 and 1996) Lange et al. [149–152] reported applications of cholesterol oxidase in "tracking cell cholesterol". Stevens et al. [157] described methods based on cholesterol oxidase for the determination of the complex pool structure of rat adrenal mitochondria. El-Yandouzi et al. [158] determined the distribution of cholesterol in the plasma membrane of

epithelial cells using renal brush border vesicles as a model.

Another interesting study on membrane disruption or lysis by COD was described by Ghoshroy et al. [163] in 1997. The "active site lid" mechanism — a phrase coined by Li et al. [100] — was employed to explain the effect of the leakage of carboxyfluorescein marker dye from 100 nm synthetic vesicles. Two forms of COD have been used: wild type *Streptomyces* sp., which forms the Δ^4-3-ketone product, and a mutant type *E361Q*, which froms only the Δ^5-3-ketone (20 times more slowly) from the cholesterol on the surface of the vesicles. Both wild and mutant enzymes bind on to the surface of the vesicles using the "active site lid" mechanism, but only the wild type results in dye leakage and therefore membrane disruption. It can be concluded that it is the formation of the Δ^4-3-ketone (by the wild enzyme) in the membrane as opposed to the Δ^5-3-ketone (by the mutant enzyme) that causes expansion of the membrane lipid bilayer leading to the increase in its permeability. The physical attachment of the enzymes to the lipid bilayer is not in itself the cause of the leakage of the dye.

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