Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 705–713. Pergamon Press. Printed in Great Britain

# MINI-REVIEW

# CHOLESTEROL OXIDASES: PROPERTIES AND APPLICATIONS

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

(*Received* 8 *March* 1976)

## INTRODUCTION

The ability of soil micro-organisms to oxidize cholesterol has been known for many years. Turfitt demonstrated the formation of 4-cholesten-3-one from cholesterol by *Proactinomyces erythropolis* (*Nocardia erythropolis*) and showed that this was the first step in the catabolism of the sterol [1–3]. Schatz *et al.* [4] isolated a soil *Mycobacterium* which was also active in oxidising cholesterol, and this organism was studied by Stadtman *et al.* [5] who isolated 4-cholesten-3-one from the incubation of a cell-free extract with cholesterol. Dialysis of the cell-free extract resulted in only a slight loss of activity, which could not be restored by the addition of $NAD^+$ or $NADP^+$. Since the time of these investigations the oxidation of cholesterol and other 3$\beta$-hydroxysteroids has been reported many times. References to original papers and reviews are cited in the comprehensive report by L. L. Smith on microbiological reactions with steroids [6]. While in some instances the enzymes involved are true NAD-dependent dehydrogenases, in other cases—especially in oxidations of sterols—the enzymes are aptly termed oxidases [7], requiring only molecular oxygen for their action.

Flegg [8] and Richmond [9] were among the first to suggest using a cholesterol oxidase [EC 1.1.3.6] from micro-organisms as a basis for a specific assay for serum cholesterol. In Flegg's work, suitable bacteria were screened, and *Nocardia erythropolis* was found to be particularly suitable if grown on cholesterol as the sole carbon source. An ammonium sulphate precipitated protein fraction of the ultrasonically disrupted cells was used to determine serum cholesterol following saponification of cholesterol esters. The 4-cholesten-3-one produced was extracted and estimated by its U.V. absorption ($\lambda_{max.}$ 240 nm) as a conjugated enone. It was suggested that if the enzyme was an oxidase, cholesterol could be determined in terms of the hydrogen peroxide produced. A separate investigation using a preparation from another *Nocardia* species (NCIB 10554, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland) was reported by Richmond [9,10]. This enzyme was purified and shown to be a true oxidase producing one mol of hydrogen peroxide per mol of cholesterol oxidised (Fig. 1). By estimating the hydrogen peroxide, the author laid the foundations for the enzymic determination of serum cholesterol, and various analytical procedures have been commercially developed. In contrast to the above-mentioned intracellular or membrane-bound enzymes, an extracellular cholesterol oxidase was purified from the broth-filtrate of *Streptomyces violascens* by Fukuda *et al.* [11]. Uwajima *et al.* [12,13] isolated a crystalline 3$\beta$-hydroxysteroid oxidase from the culture fluid of *Brevibacterium sterolicum*. Cholesterol oxidases from various sources are becoming of increasing importance in the clinical estimation of cholesterol, and the current literature on the enzymes is surveyed in more detail below under five general classifications:—

Isolation of enzymes
Physical properties
Substrate specificity
Clinical determination of cholesterol
Selective oxidation of hydroxysteroids

*Isolation of enzymes.* The initial partial purification of cholesterol oxidase from *Nocardia erythropolis* by

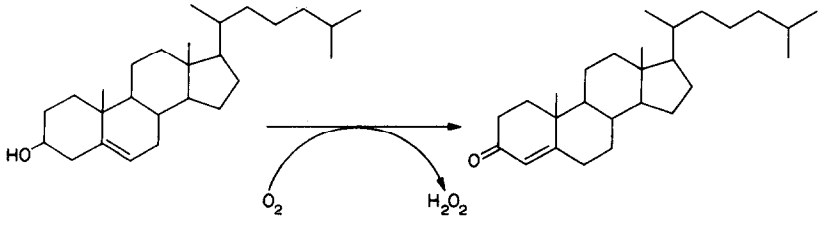


Fig. 1. The oxidation of cholesterol to 4-cholesten-3-one as catalysed by cholesterol oxidase.

Flegg [8] involved a precipitation of the cell-free extract in 30% aq. ammonium sulphate. The purification procedures for the enzyme from commercial sources are not readily available. Richmond showed by electron microscopy that the degree of release of the enzyme from the *Nocardia* sp. (NCIB 10554) during extraction procedures was related not to the fragmentation of the cells, but to damage to the cell surface, suggesting that the enzyme is closely associated with cell membrane [10]. Triton X-100 (1%) was found to release the cholesterol oxidase in yields equivalent to as much as 70% of the activity of the total cells. The enzyme was further purified by anion-exchange chromatography on DEAE–cellulose to remove catalase: elution was effected with a gradient of tris(hydroxymethyl)aminomethane chloride buffer (pH 8.0). (This purification system was found to be simpler and more efficient than a procedure based on mechanical disruption of the cells, followed by substrate-affinity chromatography using Sephadex LH-20 swollen in an ethanolic solution of cholesterol.) A large scale aerobic process for production of the enzyme has been developed by Buckland *et al.* [14]. The initial yield of the enzyme from the cells was considerably increased by improved growth conditions, including the addition of cholesterol as an emulsion in Tween 20 to give a final concentration of 2 g/l of culture. The enzyme was extracted from the cells with Triton X-100 as before, and further purified by chromatography on DEAE–cellulose, using a stepwise elution with 50 mM potassium phosphate buffer (pH 7.5) containing 0.5% of Triton X-100. A 400-fold purification in 26% yield was obtained by this method.

The extracellular enzyme isolated from *Streptomyces violascens* H 82 N-SY 7 was partially purified by precipitation with saturated ammonium sulphate solution followed by dialysis and lyophilisation [11]. 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.

The 3$\beta$-hydroxysteroid oxidase discovered in the culture fluid of *Brevibacterium sterolicum* was extensively purified by Uwajima *et al.* [12]. The broth supernatant was concentrated 5-fold, and following fractionation with ammonium sulphate, a precipitate obtained was dialysed and purified by anion-exchange chromatography on DEAE–cellulose equilibrated with 0.01 M phosphate buffer (pH 7.0). This last step removed contaminating 17$\beta$-hydroxysteroid dehydrogenase. Following further chromatography on hydroxyapatite and Sephadex G-75, the 3$\beta$-hydroxysteroid oxidase was crystallised from ammonium sulphate solutions. The purity of the crystals was established by observation of one band during gel electrophoresis, and a single peak sedimenting during ultracentrifugation. A yield of 21% after crystallisation was reported, with a 1200-fold purification compared to the culture filtrate.

*Physical properties*

(a) Nocardia *spp.* There is little published information on the physical properties of cholesterol oxidase from *Nocardia* species. The enzyme appears to have a broad pH optimum with a maximum rate observed at pH 7.0 [10, 15–17]. A temperature optimum at 32°C was observed by Brooks and Smith [18]. The thermal stability of cholesterol oxidase from *Nocardia* allows it to be used at temperatures as high as 50°C [10]. Full activity is retained for many months if the enzyme is stored at 5°C.

(*b*) *Brevibacterium sterolicum.* The physical properties and nature of the crystalline 3$\beta$-hydroxysteroid oxidase obtained from *Brevibacterium sterolicum* culture fluid have been examined in some detail by Uwajima *et al.* [13]. The enzyme crystallised as yellow rods, and the sedimentation coefficient in water at 20°C was estimated to be 3.3 S with a molecular weight of 32,500. Sephadex G-75 gel filtration indicated a mol. wt of 33,000. The isoelectric point of the enzyme was calculated to be pH 8.9. A total of 258 amino acid residues was estimated to be present per molecule of protein: there was a high proline content, while alanine and tryptophan were absent. The presence of FAD as a prosthetic group of the enzyme was indicated by the absorption spectrum. The FAD was obtained by dissociation of the enzyme by treatment at 100°C for 3 min, and identified by its absorption spectrum and by paper chromatography. The cofactor served as a prosthetic group for the oxidase apoprotein prepared by the method of Massey and Swoboda [19] in the ratio of one mol of FAD per mol of protein. No metal ions could be detected, and the 3$\beta$-hydroxysteroid oxidase was unaffected by chelating reagents. Silver and mercury compounds and *p*-chloromercuribenzoate caused inhibition, though this could be almost completely prevented by glutathione. Like the enzymes isolated from *Nocardia* spp., this enzyme was highly stable: it possessed a broad pH optimum around pH 7.5. Full activity was retained after 30 min at 50°C in 0.1 M phosphate buffer of pH 7.0. The enzyme was also stable for 30 min at 37°C over the pH range 4–10. Properties of the enzyme from *Streptomyces violascens* do not appear yet to have been reported in full [11].

*Substrate specificity*

The substrate specificities of cholesterol oxidases from various sources have been studied in some detail. The enzymic reaction probably consists of an oxidation step followed – in the case of 5-ene-steroids – by isomerisation of the $C_5$–$C_6$ double bond to the $C_4$–$C_5$ position. Some investigations have involved estimating the relative rates of conjugated enone formation by the increase in U.V. absorption. However, this is a measure of both steps, and, moreover, cannot be applied to the oxidation of 5$\alpha$-3$\beta$-hydroxysteroids. The rate of the oxidation step can be measured by monitoring oxygen uptake or

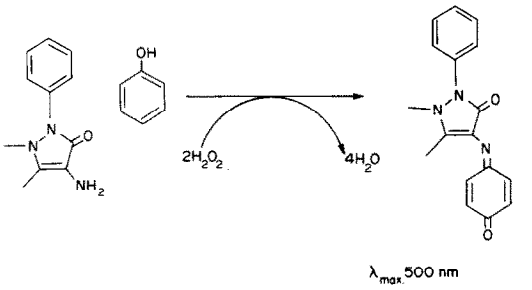


Fig. 2. The assay of hydrogen peroxide, by the oxidative coupling of phenol and 4-aminoantipyrine catalysed by horseradish peroxidase [21].

hydrogen peroxide production, and both methods have been applied to the enzymes from *Nocardia* spp. and *Brevibacterium sterolicum*.

(a) *Cholesterol oxidase from* Nocardia *spp.* Flegg [8] in her studies with *N. erythropolis* used 2,4-dinitrophenylhydrazine to detect ketone formation, and also measured the absorption at 240 nm where appropriate. 5α-Cholestan-3β-ol, 5,7-cholestadien-3β-ol, 5,7,*E*-22-ergostatrien-3β-ol (ergosterol) and (20*S*)-5-cholestene-3β,20-diol (20α-hydroxy-cholesterol) were found to be oxidised by cholesterol oxidase though all but the last were oxidised at a slower rate than cholesterol. Richmond [10] 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 5-ene-3β-hydroxy-steroids 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 5β-cholestan-3β-ol were recorded as remaining unoxidised: however, the U.V. assay system would not have revealed the formation of a saturated 3-ketone. A $K_M$ for cholesterol of $1.4 \times 10^{-5}$ M was determined. Using the same enzyme source, Allain *et al.* [20] 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 32–60% of the rate for cholesterol. In these experiments, the hydrogen peroxide generated was measured by the oxidative coupling of 4-aminoantipyrine with phenol by horse-radish peroxidase to give a quinone-imine absorbing at 500 nm [21] (Fig. 2).

Table 1. Substrate specificity of cholesterol oxidase from *N. erythropolis* as determined by Wortberg [22]

| | Relative rates* |
|---|---|
| Cholesterol | 100 |
| Desmosterol | 98 |
| 5α-Cholestan-3β-ol | 87 |
| 4-Cholesten-3β-ol | 75 |
| 5-Cholesten-3α-ol | 0 |
| Campesterol | 67 |
| Sitosterol | 50 |
| Stigmasterol | 22 |
| Ergosterol | 18 |
| 7,*E*-22-Ergostadien-3β-ol | 26 |
| Lanosterol | 0 |

* Initial relative reaction rates were determined using peroxidase and 2,2′-azino-di-[3-ethyl-benzthiazoline sulphonate] [23].

The cholesterol oxidase isolated from *N. erythropolis*, as supplied by Boehringer–Mannheim, has been the subject of two independent studies of substrate specificity. Wortberg [22] has determined 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, relative rates of ketone formation were measured by U.V. spectroscopy or by g.l.c. [17]: more detailed work was later based on measurements of hydrogen peroxide production using the peroxidase-chromogen system of Trinder [21] as previously used by Allain *et al.* (Fig. 2) [20]. Sterols were solubilised by addition as isopropanol solutions to the phosphate buffer containing Triton X-100. Kinetic constants ($K_M$ and $V_{max.}$) were determined and are summarised in Table 2. A $K_M$ value of 2.9 μM for cholesterol was calculated as compared to a reported value of approximately 1 μM [16]. 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 2) may be 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.

Wortberg observed no oxidation of a natural mixture of 4-methylsterols, or of lanosterol [22]. The adverse effect of a 4-methyl group is illustrated in Table 2. 4,4-Dimethylcholesterol and 4α-methylcholesterol were very slowly oxidised, though 4β-methylcholesterol was a slightly better substrate [24]. A similar difference in the rates of oxidation has been noted with 4α- and 4β-hydroxycholesterols [27] and may be associated with the elimination of a 3α-hydrogen atom during oxidation. A 4α-substituent might therefore be expected to have a greater hindering effect than its 4β-epimer. Although 5α-cholestan-3β-ol

Table 2. Apparent $K_M$ and relative $V_{max.}$ values [18, 24] for the oxidation of some 3-hydroxysteroids by cholesterol oxidase from *N. erythropolis*

| 3-Hydroxysteroid | $K_M$ ($\mu$M) | 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-Cholest-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 (25*R*) | 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 | | * |
| 5-Cholesten-3α-ol | | * |
| 4-Cholesten-3α-ol | | * |
| 5β-Cholestan-3α-ol | | † |
| Cholecalciferol | | † |
| Solasodine | | * |
| Solanidine | | * |

* Detectable rate but too low for kinetic measurements.
† No detectable oxidation.

Steroids dissolved in propan-2-ol (50 $\mu$l) 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 peroxide (0.18 U) which oxidatively coupled the 4-aminoantipyrine and phenol to give a quinoneimine absorbing at 500 nm [21]. Provisional values of $K_M$ and $V_{max.}$ were obtained by the graphical method of Eisenthal and Cornish-Bowden [25] and were refined by using a computer program for a least-squares adjustment of the Michaelis–Menten curve [26]. Standard deviations have been omitted from this table.

was oxidised at a rate approximately 80% that of cholesterol, the 5β-isomer (coprostanol) was unaffected by the enzyme. Extremely low oxidation rates were observed for the 3α-hydroxy epimers of cholesterol and of 4-cholesten-3β-ol [24].

The effect of variations of the side-chain on the reactivity of the steroid [17, 18, 24] is also illustrated in Table 2. Sitosterol was readily oxidised [22]. Introduction of a $C_{22}$–$C_{23}$ or $C_{24}$–$C_{28}$ double bond decreased the oxidation rate. Diosgenin was slowly oxidised, but the steroidal alkaloids solanidine and solasodine were extremely poor substrates. A study of hydroxylated cholesterols indicated that the terminal substituent in 26-hydroxycholesterol considerably decreased the rate of oxidation of the 3β-hydroxy group, whereas a 24-hydroxyl group stimulated the reaction. The promotive effect of a polar group at $C_{24}$ was also noted with 24-ketocholesterol.

Preliminary evidence [10, 17] that the length of the $C_{17}$ chain was an important factor in determining reaction rate, was confirmed in the kinetic studies. A series of 3β-hydroxysteroids with hydroxylated side-chains containing 0 8 carbon atoms was employed, and the correlation of $V_{max.}$ against side-chain length is illustrated in Fig. 3. An interesting feature is the higher rate of reaction of 5-cholene-3β,24-diol than of 26-hydroxycholesterol—the favourable hydroxyl group position compensating for the reduced side-chain. The $K_M$ values in this series reflect no major differences in the degree of binding. The larger variations in $V_{max.}$ suggest that the longer side-chains serve to orientate the bound steroid so that the 3β-hydroxyl group and the active site are suitably aligned. Thus 17β-(hydroxymethyl)-5-androsten-3β-ol, 5-androstene-3β,17β-diol and dehydroepiandrosterone were very poor substrates. Derivatives of 17-hydroxy- and 17-ketosteroids, simulating in some degree the side-chains of the sterols, were examined. The ester group in 5-androstene-3β,17β-diol 17-benzoate did not serve as an efficient substitute for the cholesterol side-chain; neither did the 16,17-*n*-butaneboronate group in the cyclic boronate of 5-androstene-3β,16β,17β-triol [18]. Substituted oximes of dehydroepiandrosterone were also exam-

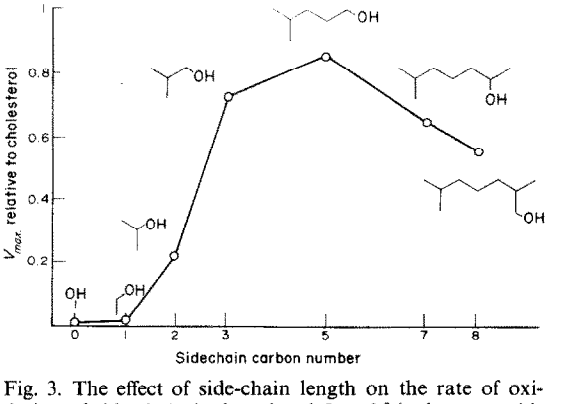


Fig. 3. The effect of side-chain length on the rate of oxidation of side-chain hydroxylated 5-en-3β-hydroxysteroids by cholesterol oxidase (*Nocardia erythropolis*).

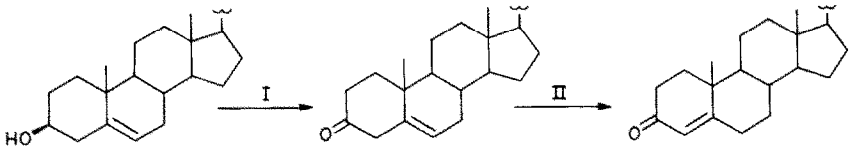


Fig. 4. Presumed sequence of steps for the conversion of cholesterol to 4-cholesten-3-one by cholesterol oxidase. I. Oxidation; II. Isomerisation.

ined as possible substrates: among these, the 17-isopentyloxime and 17-benzyloxime derivatives were oxidised at rates comparable to those of sterols (Table 2). However, a ten-fold increase in $K_M$ value was observed and can probably be attributed to an adverse effect of the nitrogen atom (cf. the high $K_M$ and low reaction rate observed with 20,25-diazacholesterol). Cholesterol oxidase did not oxidise cholecalciferol: it may be noted that this *seco*-sterol has been proposed as a substrate for other enzymes acting on cholesterol [28].

(b) *Extracellular cholesterol oxidases.* The cholesterol oxidase isolated from *Streptomyces violascens* broth filtrate was tested for its substrate specificity by a qualitative chromatographic method [11]. In addition to cholesterol, the following steroids were oxidised: epiandrosterone, dehydroepiandrosterone, 4-androstene-3$\beta$,17$\beta$-diol, 5-androstene-3$\beta$,17$\beta$-diol, pregnenolone, 5$\alpha$-pregnane-3$\beta$,16$\beta$,20$\alpha$-triol, 5$\alpha$-cholestane-3$\beta$,5$\alpha$-diol and 5$\beta$-cholestan-3$\beta$-ol. However, 3$\alpha$-hydroxysteroids, oestrogens and lanosterol were not oxidised.

The crystalline cholesterol oxidase isolated from the culture supernatant of *Brevibacterium sterolicum* by Uwajima *et al.* was incubated with a number of steroids and the relative enzymic oxidation rates were calculated by determining oxygen uptake manometrically [13]; typical results were: cholesterol (100), dehydroepiandrosterone (41), pregnenolone (22), sitosterol (20), 5$\alpha$-cholestan-3$\beta$-ol (13) and stigmasterol (10). Ergosterol, diosgenin, cholecalciferol, cholic acid, digitoxigenin, 5$\alpha$-androstane-3$\alpha$,17$\beta$-diol, androsterone, testosterone and oestradiol did not serve as substrates. The relative rates reported differ markedly from those observed with cholesterol oxidase from *Nocardia* spp.

*Mechanism of action.* Little is so far known of the mechanism of action of cholesterol oxidases. Richmond demonstrated 90% of the theoretical yield of hydrogen peroxide from the oxidation of cholesterol, assuming that one mol is produced per mol of 4-cholesten-3-one formed [10]. Similar results have been reported by Uwajima *et al.*, for the extracellular enzyme from *Brevibacterium sterolicum* [13].

The oxidation of cholesterol by cholesterol oxidases probably involves an oxidative step giving 5-cholesten-3-one, followed by isomerisation to 4-cholesten-3-one (Fig. 4). The 3$\beta$-hydroxysteroid dehydrogenase and 5-ene-3-ketosteroid isomerase from *Pseudomonas testosteroni* have been separated, crystallised and extensively studied by Talalay and his co-workers [29, 30]. However, no separation of the components of cholesterol oxidase has been reported. The 3$\beta$-hydroxysteroid oxidase from *Brevibacterium sterolicum* has been shown to contain FAD, and Uwajima *et al.* [13] have suggested that this is the active moiety for the oxidation of the 3$\beta$-hydroxyl group. The reduced FAD formed would then be oxidised by molecular oxygen to give hydrogen peroxide, perhaps by a mechanism similar to that proposed for glucose oxidase [31]. The isomerisation was considered either to be nonenzymic or to occur by further action of the 3$\beta$-hydroxysteroid oxidase. There appears to be no published evidence for the presence of FAD in the cholesterol oxidases of *Nocardia* spp. These enzymes are not dehydrogenases since molecular oxygen is required, and the addition of $NAD^+$ or $NADP^+$ has either no effect [5] or is slightly inhibitory [27].

Preliminary observations have been made on the isomerising activity of the cholesterol oxidase of *N. erythropolis* [27]. 5-Cholesten-3-one was isomerised only about one-third as fast as 5-androstene-3,17-dione, whereas the rate of oxidation of cholesterol was faster (by two orders of magnitude) than that of dehydroepiandrosterone. The $K_M$ and $V_{max.}$ values for the isomerisation steps were much higher than for the accompanying oxidations. The $K_M$ value for 5-androstene-3,17-dione is of the same order as that reported for the 5-ene-3-ketosteroid isomerase of *Pseudomonas testosteroni* [32]. The oxidising and isomerising activities of cholesterol oxidases may not reflect the presence of separable enzymes. It may be noted that the 3$\beta$-hydroxysteroid dehydrogenase/5-ene-3-ketosteroid isomerase complex of adrenal microsomes has so far defied separation into two components, and terminal amino acid analyses suggest the presence of only one type of peptide chain [33].

Specificity studies of enzymes with lipid substrates require cautious interpretation, because of the uncertain accessibility of the enzyme to different compounds. The investigations of cholesterol oxidase that have been described employed ethanol or propan-2-ol together with Triton X-100 as solubilizing agents [8, 10, 13, 18, 22, 24]. The 5-ene-3-ketosteroid isomerase of *P. testosteroni* has been reported as possessing no activity towards 5-cholesten-3-one (solubilised with methanol) [32]. However, Jones and Wigfield [34] have suggested that this may be a result of micelle formation and that under appropriate conditions the $C_{27}$ steroid might be as good a substrate as 5-androstene-3,17-dione.

Table 3. Methods used for the enzymic determination of serum cholesterol

| Outline of procedure | $\lambda_{max.}$ nm | References |
|---|---|---|
| 1. Direct U.V. of enone | 240 | 8, 16, 47 |
| 2. Determination of $H_2O_2$ | | |
| (a) Ti(IV): xylenol orange | 550 | 10 |
| (b) peroxidase: 2,2'-azino-di-[3-ethyl-benzthiazoline sulphonate] | 418 | 48 |
| (c) peroxidase; phenol; 4-aminoantipyrine | 500–520 | 20, 42, 43, 49–56 |
| (d) peroxidase; *o*-dianisidine | 440 | 15, 57, 58 |
| (e) catalase; methanol; $NH_4^+$; acetylacetone | 405 | 41, 59–61 |
| (f) catalase; methanol; 2-hydrazono-2,3-dihydro-3-methylbenzthiazole HCl | 630 | 56 |
| (g) as (e) with fluorimetric estimation | (485*) | 44 |
| (h) peroxidase; homovanillic acid | (425†) | 45 |
| (i) iodide ion oxidation, monitored potentiometrically | | 46 |
| (j) [unspecified in abstract] | | 62–64 |

* Fluorescence emission (excitation at 405 nm).
† Fluorescence emission (excitation at 315 nm).

*Clinical determination of cholesterol*

The estimation of serum cholesterol concentration is a common and important assay in clinical biochemistry. It is significant, in particular, for the assessment of arteriosclerosis and other lipid disorders, and of the risk of thrombosis and myocardial infarction. Chemical analyses have been very extensively used despite a number of drawbacks [35–38]: for example, they are subject to interference from other serum constituents (haemoglobin, bilirubin, etc.) and they require corrosive reagents. The demonstration [8, 9] that preparations from *Nocardia* spp. containing cholesterol oxidase could be used to measure serum cholesterol, laid the foundation for further development of this more specific method. In Flegg's procedure [8], the 4-cholesten-3-one produced was assayed by its U.V. absorption following solvent extraction of the incubation mixture. Cholesterol esters were hydrolysed by initial saponification. Though this scheme gave a good correlation with the Liebermann–Burchard method, it suffered from the disadvantages of long incubation times (up to 2 h) and the necessity for solvent extraction of 4-cholesten-3-one. Similar methodology was employed in the early methods of the Boehringer–Mannheim group. Richmond [10] estimated hydrogen peroxide production during cholesterol oxidation, using quadrivalent titanium and xylenol orange to give a complex absorbing at 550 nm [39]. Cholesterol esters were hydrolysed by alkali, and mercuric ions were subsequently added to oxidise substances formed during this saponification. This unfortunately caused some inhibition of the cholesterol oxidase. Allain *et al.* [20] used cholesterol ester hydrolase [EC 3.1.1.13] to hydrolyse the cholesterol esters. The hydrogen peroxide produced was estimated by using horse-radish peroxidase to effect the oxidative coupling of 4-aminoantipyrine with phenol, affording a quinone-imine absorbing at 500 nm (Fig. 2) [21]. Little interference was observed from urea, ascorbic acid, creatinine, glucose, bilirubin, uric acid, haemoglobin or sodium bromide. The method was highly reproducible: the results with serum samples agreed very well with those obtained by two automated procedures and by the method of Abell *et al.* [40]—all based on the Liebermann–Burchard reaction. The minor sterols present in normal human serum caused less interference when the enzymic method was used. Röschlau *et al.* [41] have developed serum cholesterol determinations based on measuring the hydrogen peroxide produced by its reaction with methanol to give formaldehyde in the presence of catalase. Further reaction with $NH_4^+$ and acetylacetone then gave 3,5-diacetyl-1,4-dihydrolutidine, which was estimated by its U.V. absorption at 405 nm. Alkaline hydrolysis of cholesterol esters [10] has been employed by BDH [42]. Thiols arising as by-products, which would have caused reduction of hydrogen peroxide, were removed by oxidation with iodate. Evidence was presented for the stability of cholesterol during saponification, and it was implied that this method might be superior to enzymic hydrolysis, the efficiency of which varies for different esters. However, the need for neutralisation prior to enzymic oxidation is an obvious disadvantage.

The determination of serum cholesterol by the above methods, or variants thereof (Table 3) gave extremely good correlation with classical methods and was far less susceptible to interference from haemoglobin, bilurubin, ascorbic acid, glucose etc. The use of cholesterol oxidase also has a further great advantage in that the concentrations of free and esterified sterol can be readily estimated. The enzymic procedures have been satisfactorily adapted for use with automatic analysers. An independent study [43] of a typical commercially-developed procedure showed that it gave a very good correlation with established methods, together with high precision. The response in the enzymic method was linear up to a cholesterol concentration of 430 mg/dl of serum—a higher level than in other procedures. Minor interference by bilirubin was observed. Haemolysed and lipaemic sera presented no difficulty; nor did the presence of

common steroid drugs, including cortisone acetate and dexamethasone.

An alternative method of estimation, suitable for automatic analysis, has been based on the fluorimetric determination of 3,5-diacetyl-1,4-dihydrolutidine (produced from hydrogen peroxide as outlined above): measurements were made at 485 nm, with excitation at 405 nm [44]. In another procedure, peroxidase was used to catalyse the oxidation of homovanillic acid to 2,2'-dihydroxy-3,3'-dimethoxydiphenyl-5,5'-diacetic acid, which was determined by its fluorescence at 425 nm [45].

Papastathopoulos and Rechnitz have devised and evaluated a potentiometric (automated) method for determining the hydrogen peroxide produced by cholesterol oxidase [46]. The change in iodide concentration produced by a molybdenum(VI)-catalysed indicator reaction was employed, i.e.

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow{Mo(VI)} I_2 + 2H_2O$$

using a specially constructed flow-through membrane electrode. This non-spectrophotometric system avoids possible interference by haemoglobin and bilirubin.

In addition to the examples of the enzymic determination of serum cholesterol, cited in Table 3, some applications of cholesterol oxidase to other biological samples have been reported. The determination of cholesterol in high-density lipoprotein fractions prepared by precipitation with $Mn^{2+}$ was achieved [65] by incorporating EDTA into the reagent normally used for serum [20]. Enzymic measurements of initial cholesterol esterification in human plasma [66], and of cholesterol in bile [67], have also been described. The markedly different specificity of the enzyme from *Brevibacterium* (as compared with that from *Nocardia*) has allowed its application to the determination of urinary dehydroepiandrosterone [68].

Brief reference may be made to the potentially wider application of cholesterol oxidases, exemplified by analytical methods for sterols in foodstuffs [69] and in the unsaponifiable material of rapeseed oil and lard [22]. The use of the enzymes for analysis of mixtures of sterols clearly must be based upon a satisfactory knowledge of substrate specificity.

*Selective oxidation of hydroxysteroids*

In 1974, Uwajima, *et al.*, suggested that the 3β-hydroxysteroid oxidase from *B. sterolicum* could be used in the microanalysis of steroids in clinical specimens and foods, for the determination of the configuration of 3-hydroxy-steroids and in the preparation of 3-ketosteroids [13]. In the same year, cholesterol oxidase from *N. erythropolis* was applied to aid the identification of 26-hydroxycholesterol isolated from human brain [70], and it soon became apparent that this type of selective enzymic oxidation would have a broad range of application in the microanalytical characterisation of hydroxysteroids.

Smith and Brooks [17] used cholesterol oxidase to oxidise pairs of 5-ene- and 5α-3β-hydroxy $C_{21}$, $C_{27}$, $C_{28}$ and $C_{29}$ steroids, which are not effectively separable by g.l.c. on commonly used liquid phases unless columns of high resolving power are used. The products, 4-ene- and 5α-3-ketosteroids respectively, were satisfactorily separated, by virtue of the different retention index increments accompanying oxidation: for OV-1 stationary phase at 275°, these were approximately +115 (5-en-3β-ol→4-en-3-one) and +30 (5α-3β-ol→5α-3-one) respectively. A synthetic mixture of seven sterols which gave only three peaks during g.l.c. was resolved into seven peaks after incubation with the enzyme. One of the sterols (4,4-dimethylcholesterol) was not oxidised to any extent during the incubation period, and was thus distinguished from stigmasterol, which yielded stigmasterone. The low reactivity of androstenes with the enzyme from *N. erythropolis* was exploited to separate 5-androstene-3β, 16α, 17α-triol and 5-pregnene-3β,20β-diol, which do not separate as their TMS ethers during conventional g.l.c. on OV-1. The latter steroid was selectively oxidised during the incubation period, and the products were separable after derivatisation.

The very low reactivity of 17-keto-3β-hydroxyandrostenes was overcome by the formation of the 17-isopentyloximes to mimic the side-chain of cholesterol (Table 2). This enabled epiandrosterone and dehydroepiandrosterone to be separated by incubation of their isopentyloximes with the enzyme [18] in an analogous manner to that used for sterols and pregnenes [17]. A variety of hydroxy- and keto-analogues of dehydroepiandrosterone (19-, 18-, 15α-, 7α- and 7β-hydroxy-; 11-keto-; and D-homo-) were oxidised as their 17-isopentyloxime derivatives. However, 7-ketodehydroepiandrosterone formed a di-isopentyloxime, which was unattacked by the enzyme. This represents a further basis for distinguishing steroids, by a structural modification that suppresses reactivity. Isopentyloximes have been applied in the analysis of urinary steroid mixtures [71] and therefore a derivatised steroid mixture might contain the isopentyloximes of 20-ketopregnanes. The 20-isopentyloximes of pregnenolone and 3β-hydroxy-5α-pregnan-20-one were both shown to be oxidised, and were distinguished by g.l.c. [18]. The problem of overlapping g.l.c. peaks for the TMS ethers of 5-pregnene-3β,17α,20β-triol and of the 17-isopentyloxime of 3α-hydroxy-5α-androstane-11,17-dione was solved by incubation of the steroids with cholesterol oxidase prior to trimethylsilylation.

Cholesterol oxidase is particularly suitable for microanalytical applications in conjunction with combined g.l.c.–mass spectrometry [72]. In the course of the characterisation of 26-hydroxycholesterol [70], a novel rearrangement of a TMS group was observed in the mass spectrum of 26-trimethylsilyloxy-4-cholesten-3-one: analogous 4-ene-3-ketosteroids with hydroxylated sidechains were simply prepared with the aid of the enzyme [73]. The mass spectra of other

3-ketosteroids similarly obtained have been reported [74, 75].

Among other selective oxidations accomplished with the enzyme from *N. erythropolis*, the following are noteworthy. 19-Hydroxycholesterol gave 19-hydroxy-4-cholesten-3-one in 100% yield [27]. The 19-nor-5α-stanols from the sponge *Axinella polypoides* [76] were oxidised by the enzyme in contrast with the A-nor-5α-stanols from *Axinella verrucosa* [77] which did not serve as substrates [18]. 5,16-Pregnadien-3β,20α-ol yielded 20α-hydroxy-4,16-pregnadien-3-one [18, 74], while 18-hydroxypregnenolone was converted to 18-hydroxyprogesterone [27].

The potential of cholesterol oxidase in the preparative formation of steroids has yet to be fully exploited. Though other microbial enzymes are available for oxidising the 3β-hydroxy-group (of 5-ene-, 4-ene- and 5α-steroids), they lack the hydroxy group specificity and wide range of types of steroids oxidised, characteristic of cholesterol oxidase. This enzyme is also highly stable and requires no expensive co-factors. For oxidations on a preparative scale, it may not be necessary to present the substrate in a homogeneous system. Preparative oxidation of dehydroepiandrosterone to 5-androstene-3,17-dione, catalysed by 3(or 17)α-hydroxysteroid: $NAD^+$ oxidoreductase, has been achieved in a heterogeneous medium (butyl acetate/phosphate buffer) [78]. Application of a *Nocardia* strain (NCIB 10554) to the larger-scale conversion of cholesterol to 4-cholesten-3-one in a stirred mixture of carbon tetrachloride and water has been demonstrated by Buckland *et al.* [79]. Immobilisation of cholesterol oxidase has been investigated by the same group [80].

## CONCLUSIONS

The oxidation of hydroxysteroids is only one example of the wide range of steroid reactions that can be effected by micro-organisms. Other transformations that have been extensively studied include hydroxylation, epoxidation, dehydrogenation, and oxidative cleavage of the carbon skeleton (nuclear and side-chain) [6, 81]. Microbiological reactions are well established in the manufacture of hormonal steroids, but have only recently become practicable for general laboratory use, with the advent of purified enzymes suitable for use in place of the original organisms. The unexacting conditions required for the action of cholesterol oxidase make this enzyme particularly attractive as a laboratory reagent. The two principal forms of the enzyme currently available [*Nocardia* and *Brevibacterium* types] have many potential analytical applications in addition to those hitherto explored. Extension to preparative-scale reactions is also feasible, but would be facilitated by the general availability of immobilised forms of the enzymes.

Increasing application of other enzymic techniques is being made in the assay of steroids [82, 83] and in general steroid chemistry [6, 84]. It also seems likely that in the large-scale production of hormonal steroids, the use of immobilised enzymes may well supersede some traditional chemical and microbiological processes [85].

*Acknowledgements*—We thank Dr. F. L. Mitchell (Clinical Research Centre, Northwick Park, London) for stimulating our interest in cholesterol oxidase. We are also grateful to Dr. J. K. Grant (Department of Steroid Biochemistry, University of Glasgow) for his encouragement in the compilation of this review; and to the many colleagues who have kindly provided gifts of steroids. Research support has been provided by a project grant from the Medical Research Council to C. J. W. Brooks and Professor W. A. Harland.

## REFERENCES

1. Turfitt G. E.: *Biochem. J.* **38** (1942) 492–496.
2. Turfitt G. E.: *Biochem. J.* **40** (1946) 79–81.
3. Turfitt G. E.: *Biochem. J.* **42** (1948) 376–383.
4. Schatz A., Savard K. and Pintner I. J.: *J. Bacteriol.* **53** (1949) 117–125.
5. Stadtman T. C., Cherkes A. and Anfinsen C. B.: *J. biol. Chem.* **206** (1954) 511–523.
6. Smith L. L.: In *Specialist Periodical Reports Chem. Soc. Terpenoids and Steroids* Vol. 4 (1974) pp. 395–530.
7. *Enzyme Nomenclature*, 1972. Elsevier, Amsterdam, Netherlands.
8. Flegg H. M.: *Ann. clin. Biochem.* **10** (1973) 79–84.
9. Richmond W.: *Scand. J. clin. Lab. Invest.* **29** (1972) Suppl. 126 Abs. No. 3.25.
10. Richmond W.: *Clin. Chem.* **19** (1973) 1350–1356.
11. Fukuda H., Kawakami Y. and Nakamura S.: *Chem. Pharm. Bull.* **21** (1973) 2057–2060.
12. Uwajima T., Yagi H., Nakamura S. and Terada O.: *Agric. biol. Chem.* **37** (1973) 2345–2350.
13. Uwajima T., Yagi H. and Terada O.: *Agric. biol. Chem.* **38** (1974) 1149–1156.
14. Buckland B. C., Richmond W., Dunnill P. and Lilly M. D.: In *Industrial Aspects of Biochemistry* (Edited by B. Spencer) Vol. 2 *FEBS*. Symp. Amsterdam (1974) pp. 65–79.
15. Bennett S. J. and Willis J. E.: *Clin. Chem.* **20** (1974) 859 Abs. No. 24.
16. Cholesterol Oxidase: In *Methods of Enzymatic Analysis* (Edited by H. U. Bergmeyer). Academic Press, New York. Vol. 1 (1974) p. 440.
17. Smith A. G. and Brooks C. J. W.: *J. Chromatog.* **101** (1974) 373–378.
18. Brooks C. J. W. and Smith A. G.: *J. Chromatog.* **112** (1975) 499–511.
19. Massey V. and Swoboda B. E. P.: *Biochem. Z.* **338** (1963) 474–484.
20. Allain C. C., Poon L. S., Chan C. S. G., Richmond W. and Fu P. C.: *Clin. Chem.* **20** (1974) 470–475.
21. Trinder P.: *Ann. clin. Biochem.* **6** (1969) 24–27.
22. Wortberg B.: *Z. Lebensm. Unters.-Forsch.* **157** (1975) 333–338.
23. Bergmeyer H. U. and Bernt E.: In *Methods of Enzymatic Analysis* (Edited by H. U. Bergmeyer). Academic Press, New York, Vol. 3 (1974) pp. 1205–1214.
24. Smith A. G. and Brooks C. J. W.: *Biochem. Soc. Trans.* **3** (1975) 675–677.
25. Eisenthal R. and Cornish-Bowden A.: *Biochem. J.* **139** (1974) 715–720.
26. Wentworth W. E.: *J. chem. Educ.* **42** (1965) 96–103.
27. Smith A. G. and Brooks C. J. W.: Unpublished data.
28. Fraser D. R. and Kodicek E.: *Nature, Lond.* **220** (1968) 1031–1032.

29. Talalay P.: In *The Enzymes* (Edited by P. D. Boyer, H. Lardy and K. Myrbäck). Academic Press, New York, Vol. 7 (1963) pp. 177–202.
30. Talalay P.: *Ann. Rev. Biochem.* **34** (1965) 347–380.
31. Bentley R.: In *The Enzymes* (Edited by P. D. Boyer, H. Lardy and K. Myrbäck). Academic Press, New York, Vol. 7 (1963) pp. 567–586.
32. Kawahara F. S., Wang S.-F. and Talalay P.: *J. biol. Chem.* **237** (1962) 1500–1506.
33. Ford H. C. and Engel L. L.: *J. biol. Chem.* **249** (1974) 1363–1368.
34. Jones J. B. and Wigfield D. C.: *Can. J. Chem.* **46** (1968) 1459–1465.
35. Brown W. D.: *Aust. J. exp. Biol. med. Sci.* **39** (1961) 209–222.
36. Tonks D. B.: *Clin. Biochem.* **1** (1967) 12–29.
37. Fasce C. F. and Vanderlinde R. F.: *Clin. Chem.* **18** (1972) 901–908.
38. Brown S. S.: *Ann. clin. Biochem.* **10** (1973) 146–154.
39. Tammes A. R. and Nordschow C. C.: *Am. J. clin. Pathol.* **49** (1968) 613–621.
40. Abell L. L., Levy B. B., Brodie B. B. and Kendall F. E.: *J. biol. Chem.* **195** (1952) 357–366.
41. Röschlau P., Bernt E. and Gruber W.: *Z. Klin. Chem. Klin. Biochem.* **12** (1974) 403–407.
42. *A Manual Kit for the Enzymatic Determination of Total and Free Cholesterol.* B.D.H. Chemicals Ltd., 4th Edn. 1975.
43. Witte D. L., Barrett D. A. and Wycoff D. A.: *Clin. Chem.* **20** (1974) 1282–1286.
44. Fruchart J. C., Buassa-Bu-Tsumbu, Jaillard J. and Sezille G.: *Clin. chim. Acta* **63** (1975) 399–402.
45. Huang H., Kuan J. W. and Guilbault G. G.: *Clin. Chem.* **21** (1975) 1605–1608.
46. Papastathopoulos D. S. and Rechnitz G. A.: *Analyt. Chem.* **47** (1975) 1792–1796.
47. Röschlau P., Bernt E. and Gruber W.: In *Methods of Enzymatic Analysis* (Edited by H. U. Bergmeyer). Academic Press, New York, Vol. 3 (1974) pp. 1890–1893.
48. *Biochemica News* No. 1 (1974) The Boehringer Corporation (London) Ltd.
49. *Total Cholesterol Kit, Microbics Operations.* Beckman Instruments, Inc., Fullerton, U.S.A. (1975).
50. Evaluation of Enzymatic Determination of Cholesterol: *Lipid Bull.* **3** (1975) 1–2, Supelco Inc., Bellefonte, Pennsylvania, U.S.A.
51. *Instruction Sheets for Cholesterol Determinations using the Technicon Auto Analyzer.* The Boehringer Corporation (London) Ltd., (1975).
52. O'Bryan D. P., Engel R. H. and Harding R. S.: *Clin. Chem.* **21** (1975) 942, Abs. No. 5.
53. Lie R. F., Schmitz J., Pierre K. and Gochman N.: *Clin. Chem.* **21** (1975) 942, Abs. No. 7.
54. Wentz P. W., Savory J. and Cross R. E.: *Clin. Chem.* **21** (1975) 941, Abs. No. 2.
55. Röschlau P., Bernt E. and Gruber W.: *Clin. Chem.* **21** (1975) 941, Abs. No. 1.
56. Klose S., Greif H. and Hagen H.: *Clin. Chem.* **21** (1975) 942, Abs. No. 8.
57. Tarbutton P. N. and Gunter C. R.: *Clin. Chem.* **20** (1974) 724–725.
58. Willis J. E. and Bennett S. J.: *Technical Report, WB 732* (1974), P-L Biochemicals, Milwaukee, Wisc., 1974.
59. Stähler F., Munz E. and Kettermann R.: *Deutsch. Med. Wochenschr.* **100** (1975) 876–887.
60. Ziegenhorn J.: *Z. Klin. Chem. Klin. Biochem.* **13** (1975) 109–115.
61. Hagen A. and Ziegenhorn J.: *Clin. Chem.* **21** (1975) 941, Abs. No. 2.
62. Chow C. and Wong C.: *Clin. Chem.* **21** (1975) 991, Abs. No. 263.
63. Richmond W., Jackson M. J. and Brewer N.: *Clin. Chem.* **21** (1975) 942, Abs. No. 6.
64. Perlstein M. T., Thibert R. J. and Zak B.: *Clin. Chem.* **21** (1975) 941, Abs. No. 4.
65. Steele B. W., Koehler D. F., Azar M. M., Blaszkowski T. P., Kuba K. and Dempsey M. E.: *Clin. Chem.* **22** (1976) 98–101.
66. Patsch W. and Sailer S.: *Z. Klin. Chem. Klin. Biochem.* **13** (1975) 246–247.
67. Roda A., Festi D., Sama C., Mazzella G., Aldini R., Roda E. and Barbara L. *Clin. chim. Acta* **64** (1975) 337–341.
68. Yamaguchi Y., Kateishi K. and Hayashi C.: *Clin. chim. Acta* **66** (1976) 449–452.
69. *Food Analysis Sheet, Cholesterol.* Boehringer Mannheim GmbH, 1975.
70. Smith A. G., Gilbert J. D., Harland W. A. and Brooks C. J. W.: *Biochem. J.* **139** (1974) 793–795.
71. Baillie T. A., Brooks C. J. W., Chambaz E. M., Glass R. C. and Madani C. In *Mass Spectrometry in Biochemistry and Medicine* (Edited by A. Frigerio and N. Castagnoli Jr.). Raven Press, New York (1974) pp. 335–348.
72. Brooks C. J. W.: *Process Biochemistry* October (1974) 25–29.
73. Gaskell S. J., Smith A. G. and Brooks C. J. W.: *Biomed. Mass Spectrom.* **2** (1975) 148–155.
74. Smith A. G. and Brooks C. J. W.: *Biomed. Mass Spectrom.* **3** (1976) 81–87.
75. Smith A. G., Gaskell S. J. and Brooks C. J. W.: *Biomed. Mass Spectrom.* In Press.
76. Minale L. and Sodano G.: *J. Chem. Soc., Perkin Trans.* I (1974) 1888–1892.
77. Minale L. and Sodano G.: *J. Chem. Soc., Perkin Trans.* I (1974) 2380–2384.
78. Carrea G., Cremonesi P. and Antonini E.: *Steroids Lipid Res.* **5** (1974) 162–166.
79. Buckland B. C., Dunnill P. and Lilly M. D.: *Biotechnol. Bioeng.* **17** (1975) 815–826.
80. Lilly M. D., Cheetham P. S. J. and Dunnill P.: *Biochem. Eng.* In Press.
81. Raymond R. L. and Jamison V. W.: *Adv. Appl. Microbiol.* **14** (1972) 93–122.
82. Härkönen M., Adlercreutz H. and Groman E. V.: *J. steroid Biochem.* **5** (1974) 717–725.
83. Norymberski J. K., Stoddart N. and Wolstenholme J. B.: *Biochem. J.* **145** (1975) 47–51.
84. Suckling C. J. and Suckling K. E.: *Chem. Soc. Revs.* **3** (1974) 387–406.
85. Anonymous: *Chem. Eng. News* Feb. 25 (1974) 19–20.