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Preparation and Properties of a Cholesterol Oxidase from *Nocardia* sp. and Its Application to the Enzymatic Assay of Total Cholesterol in Serum

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I describe the characterization, extraction, and purification of a cholesterol:oxygen oxidoreductase (EC 1.1.3.6) from *Nocardia* sp. This enzyme catalyzes oxidation of cholesterol to Δ^4-cholestenone, with production of hydrogen peroxide. It is very stable, active over a wide pH range, and has a K_m of 1.4×10^{-5} mol/liter. It is highly specific for Δ^4- or Δ^5-3β-hydroxycholestanes, and may be applied to the assay of serum total cholesterol. In the procedure presented here, hydrogen peroxide is measured by reaction with quadrivalent titanium and xylenol orange. This constitutes a one-enzyme assay with stable reagents, which does not require protein precipitation and is not subject to interference from hemoglobin or bilirubin.

Additional Keyphrases: *Δ^4- and Δ^5-3β-hydroxycholestanes • enzyme kinetics • one-enzyme assay • H_2O_2 measurement • thin-layer chromatography • Δ^4-cholestenone • ion-exchange chromatography • cellular disruption • ultraviolet spectrophotometry*

The limitations of the many variants of the Liebermann-Burchard and Killani-Zak assays for serum cholesterol are well documented (*1-3*), and largely account for the poor performance of this test in the field (*4-7*). The possibility of an enzymatic method that might be free from such limitations appeared very attractive, and reports of cholesterol-oxidizing bacteria (*8-10*) indicated that a suitable cholesterol oxidase might be found.

The present work describes the preparation of a cholesterol:oxygen oxidoreductase from a species of *Nocardia* (NCIB 10554, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland), and the development of an assay for serum total cholesterol that appears to be more specific and precise than are current routine methods.

Materials, Methods, and Experimental Variables

Demonstration of Cholesterol Oxidation by *Nocardia* (NCIB 10554) and Identification of Oxidation Products

Nocardia cells (NCIB 10554) were grown with vigorous aeration in a 20-liter flask at 29 °C for 24 h. The culture medium was essentially that described by Stadtman et al. (*10*) and contained (grams per liter) $(NH_4)_2SO_4$, 2.0; $CaCl_2 \cdot 2H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; glycerol, 5.0; and neutralized bacteriological peptone (Oxoid, London, England), 2.0. The initial pH of the culture was 7.5; it decreased to 6.95 after 24 h. The cells (1.6 g, wet weight) were harvested by centrifugation; washed with a solution containing, per liter, 5.0 g of NaCl and 5.0 g of KCl; and stored as a paste at −30 °C.

Two-milliliter portions of a suspension of fresh or thawed *Nocardia* cells in potassium phosphate buffer (50 mmol/liter, pH 7.5) at a concentration of 50 mg (wet weight)/ml were placed in 20-ml screw-top bottles and warmed to 37 °C. At zero time, 100 µl of a cholesterol solution (26 mmol/liter of ethanol) was added to form a colloidal suspension of the substrate. The bottles were then flushed with oxygen, stoppered, and incubated with shaking for 10 min to 3 h, the amount of time depending on the activity of the cells. The reaction was stopped by adding 5 ml of a 1.0 molar ethanolic solution of potassium hydroxide; this also served to free any cholesterol and its oxidation products bound to the cells, before extraction.

The mixture was extracted by adding 4.0 ml of reagent-grade petroleum ether (bp 60-80 °C, free from aromatic hydrocarbons) and shaking for 1 min. Blank solutions and cholesterol standards were prepared by adding 100 µl of ethanol or ethanolic standard solutions of cholesterol to 2.0 ml of potassium phosphate buffer (50 mmol/liter, pH 7.5); these were included in the above procedure.

Twenty microliters of each extract was spotted on a thin-layer chromatographic plate (Merck, Darmstadt, West Germany; silica gel 60, 20 × 20 cm, layer thickness: 0.25 mm), which was then developed in benzene:ethyl acetate (9:1 by vol). Charring with 10-fold diluted sulfuric acid (heating at 80 °C) or staining with a 3 g/dl solution of phosphomolybdic acid in ethanol (heating at 110 °C) with subsequent visual inspection provided a semiquantitative means of measuring the rates of cholesterol utilization and the formation of oxidation products. Only one steroidal oxidation product could in practice be detected and it was found to have the same R_f value as Δ^4-cholestenone. When fluorescing plates (Merck F254) were used under the same conditions, Δ^4-cholestenone could be located as a dark spot, which could be measured with a densitometer ("Vitatron TLD 100";

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Dieren, Holland) in the fluorescence quench mode. Recovery of internal standards was 95% or better. Atmospheric oxidation of cholesterol could not be detected.

If the plates were charred or stained after measurement by fluorescence densitometry, Δ^4-cholestenone production, cholesterol utilization, and the possible formation of other steroidal products could be monitored on the same plate. Consequently, this procedure had many advantages for preliminary investigations.

Having found Δ^4-cholestenone to be the sole steroidal product of oxidation of cholesterol by the organism, we could more conveniently assay its cholesterol oxidase activity by measuring the change in absorbance of the petroleum ether extracts at 240 nm vs. appropriate blanks, with Δ^4-cholestenone standards taken through the entire procedure. This method was also used to assay homogenates and cell-free extracts during extraction and purification studies, and to follow the specific activity of the organism during culture in large fermentors (*11*). In optimizing conditions for the assay of whole cells, we found that oxygen was a limiting factor only when cell suspensions in excess of 14 mg (wet weight)/ml were used. Kinetic studies demonstrated that the initial rate of cholesterol oxidation was constant if initial cholesterol concentrations were greater than 0.5 mmol/liter with cell concentrations of 5 mg (wet weight)/ml.

Isolation and Purification of the Enzyme

Cell rupture was investigated by several conventional techniques:

1. *Grinding with solid carbon dioxide:* 7.5 g of cells were ground with an excess of crushed solid carbon dioxide for 30 min.

2. *Preparation of an acetone powder:* 5.0 g of cells were suspended in 50 ml of acetone at −30 °C. After the suspension had been stirred for 2 h, it was centrifuged and the procedure repeated. The resulting pellet was dried under reduced pressure at 4 °C.

3. *Ultrasonication:* A cell suspension (400 mg wet weight per ml) in potassium phosphate buffer (50 mmol/liter, pH 7.5) was sonicated for 30 min at 0 °C with a 100-W ultrasonic disintegrator (M.S.E., Crawley, England) at 9-μm amplitude.

4. *The LKB "X-press":* In this apparatus (LKB, Stockholm, Sweden) a cell paste (at −25 °C) is pressed to and fro through a small aperture under high pressure. The cells were subjected to five such passes.

5. *The Mickle process:* In this apparatus (Mickle, Dorking, England) a cell suspension as in 3 above was subjected to high-frequency vibrations in the presence of small glass beads for 1 h at 4 °C.

The suspensions from processes 3 and 4 were centrifuged at 100 000 × *g* for 1 h to yield the corresponding supernatant fractions. The cell debris from processes 1, 2, and 5 were suspended in potassium phosphate buffer (50 mmol/liter, pH 7.5) in a concentration equivalent to 100 mg (wet weight) of cells per milliliter, and extracted by stirring at 4 °C for 30 min and centrifuging at 100 000 × *g*. Typical results of these isolation procedures are summarized in Table 1.

Table 1. Cholesterol Oxidase Activity Found after Various Techniques for Disintegrating *Nocardia* sp. Cells

Isolation process	Activity recovered in homogenate (% of total)	Distribution of homogenate activity	
		% in cell debris	% in soluble fraction
Grinding with solid carbon dioxide	95	92	8
Acetone powder	52	76	24
Ultrasonication	28	75	25
LKB X-press	48	37	63
Mickle process	33	33	67

Comparison of techniques for release of enzyme. The cell debris from the rupturing techniques was negatively stained with sodium silicotungstate (4 g/dl) (*12*) and examined in a Phillips EM300 electron microscope to determine whether the different mechanical stresses that had been applied to the cells could be related to the type of damage observed and the resulting release of enzyme. Neither sonication nor grinding with solid carbon dioxide released much of the enzyme, and tended to fragment the cells with little apparent damage to the cell wall (Figure 1). The Mickle process, in contrast, released more of the enzyme with very little fragmentation but with a suggestion of superficial damage to the cell wall (Figure 2). From this we speculated that the enzyme is situated at the surface of the cell and that techniques usually used to isolate enzymes from mitochondrial membrane-bound complexes might be equally effective in this situation (*13*). Thus we investigated extraction with detergent and developed a simple process involving the non-ionic detergent "Triton X-100" (BDH Chemicals Ltd., Poole, England), the optimal conditions being:

Fifty grams (wet weight) of cells was suspended in 45 ml of tris(hydroxymethyl)aminomethane hydrochloride buffer (50 mmol/liter, pH 8.0) containing "Triton X-100" (10 ml per liter). Vigorous stirring at 20 °C for 1 h and centrifugation at 10 000 × *g* for 30 min produced a clear supernate with high specific activity, containing 70% of the total activity of the whole cells. In a typical experiment, cells capable of oxidizing 1.8 μmol of cholesterol per minute per gram wet weight were processed in this way to yield a solution containing 100 μg of protein per milliliter, with a specific activity of 1.2 μmol of cholesterol oxidized per milligram of protein per minute at pH 8.0 and 37 °C.

Removal of contaminating catalase. The Triton X-100 extract contained catalase (EC 1.11.1.6) as an impurity, which was removed by anion-exchange chromatography as follows:

Fig. 1. Electronmicrograph of *Nocardia* after sonication, showing fragments of cells with little apparent damage to the cell wall

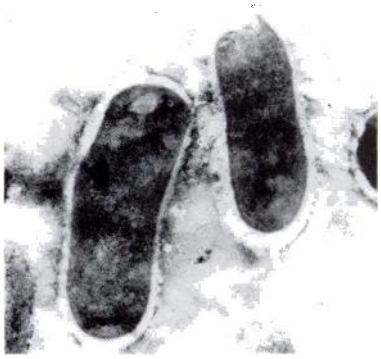

Fig. 2. Electronmicrograph of *Nocardia* after the Mickle process, showing little fragmentation of the cells but some evidence of superficial damage to the cell wall

Fifty grams of the exchange resin "DE52" (preswollen microgranular; Whatman, Maidstone, England) was equilibrated with tris(hydroxymethyl)-aminomethane hydrochloride buffer (10 mmol/liter, pH 8.0) containing 10 ml of Triton X-100 per liter, and packed in a 2.0-cm (i.d.) column to a bed height of 3.0 cm. Five milliliters of the cell extract was applied, and stepwise elution was carried out with 15-ml portions of tris(hydroxymethyl)aminomethane chloride buffer (pH 8.0) in the sequence of molarities 0.01, 0.1, 0.2, 0.3, 0.4, and 0.5 mol/liter. Five-milliliter fractions were collected at a flow rate of 1.1 ml/min. Of the total cholesterol oxidase activity, 50% was eluted in the second and third fractions collected during elution with 0.3 mol/liter buffer. These fractions were free of catalase activity, which remained bound to the resin, and the cholesterol oxidase activity was increased fourfold by this procedure.

A large-scale process based on these studies has been developed. Culture of the organism has also been optimized and the cholesterol oxidase activity of the organism increased by induction with cholesterol (*11*).

The crude enzyme preparations obtained from mechanically ruptured cells presented a more difficult purification problem, which was solved by using a form of substrate-affinity chromatography. "Sephadex LH-20" (Pharmacia, Uppsala, Sweden) was swollen in a saturated ethanolic solution of cholesterol. A slurry prepared in this manner was packed into a column, which was then washed with distilled water to precipitate cholesterol throughout the gel. It was then equilibrated with potassium phosphate buffer (50 mmol/liter, pH 7.5). Crude cell extract was applied to the column and eluted with the same buffer. Most of the protein was eluted immediately, but cholesterol oxidase, retarded by its affinity for cholesterol trapped in the gel, was eluted later. With this technique, a 100-fold purification could be achieved. Purification by detergent extraction or ion-exchange proved, however, to be much simpler and it was uneconomic to pursue mechanical disruption and substrate affinity chromatography on a large scale.

Characterization of the Enzyme

It was considered essential to establish the nature of the hydrogen acceptor involved in the enzymatic oxidation of cholesterol to Δ^4-cholestenone.

The enzyme was precipitated from cell-free extracts at 52% saturation with ammonium sulfate (at 4 °C) without undue loss of activity. This suggested that loosely bound nicotinamide coenzymes were not involved. Furthermore, using a Clark oxygen electrode (Radiometer, Copenhagen, Denmark), we demonstrated oxygen consumption during the oxidation of cholesterol by cell-free extracts, and it seemed likely that the enzyme was an oxygen oxidoreductase, which would produce hydrogen peroxide as a reaction product.

This was confirmed with both intact cells and cell-free extracts by using a peroxidase/4-aminophenazone/phenol system (*14*) to detect hydrogen peroxide. Interference from catalase activity in these unpurified preparations was anticipated and avoided by using a reagent with high peroxidase activity and containing sodium azide, a potent catalase inhibitor.

Typical experimental details were as follows:

Washed *Nocardia* cells were suspended to a concentration of 10 mg (wet weight) per milliliter in a reagent containing, per liter, 400 mg of 4-aminophenazone, 200 mg of peroxidase ("R.Z.O.6"; Hughes and Hughes, Romford, England), 1.2 g of phenol, and 1.0 g of sodium azide. Portions of the cell suspensions (2.0 ml) were placed in 20-ml vials; to the test, 0.1 ml of a 10 g/liter solution of cholesterol in ethanol was added while 0.1 ml of ethanol was added to the control tube. The bottles were flushed with oxygen, stoppered, and incubated at 37 °C for 30 min with shaking. A deep pink color developed in the test supernate, but only a faint pink color in the control. Using purified enzyme preparations, under the conditions specified for the assay of serum cholesterol, we demonstrated that 90% of the theoretical yield of hydrogen peroxide was obtained from cholesterol standards in isopropanol, assuming that one molecule of hydrogen peroxide is produced for each molecule of cholesterol oxidized.

Assay of Enzyme Activity

The insolubility of cholesterol and of Δ^4-cholestenone caused difficulty in the development of direct spectrophotometric assays for both cholesterol oxidase and serum cholesterol. We found, however, that cholesterol could be satisfactorily solubilized by adding an ethanolic solution of cholesterol to an aqueous solution of Triton X-100, and that the Δ^4-cholestenone produced by enzymatic oxidation remained in solution. The rate at which cholesterol was converted to Δ^4-cholestenone could then be followed directly by measuring the rate of increase in absorbance at 240 nm. (The contribution of hydrogen peroxide to the absorbance change at this wavelength is negligible.)

The following procedure for the assay of cholesterol oxidase was suitable for enzyme preparations that had been adjusted to an activity of about 2.0 U/ml (one unit of activity = 1 μmole of cholesterol oxidized per minute under the conditions given):

To 3.0 ml of phosphate buffer (0.1 mol/liter, pH 7.0) containing 0.5 ml of Triton X-100 per liter, at 30 °C in a cuvette of 10 mm lightpath, add 50 μl of the enzyme preparation and mix by inversion. In a double-beam spectrophotometer set at 240 nm, zero against buffered Triton X-100, add 50 μl of cholesterol in isopropanol (6.0 mmol/liter), and mix again. Measure the change of absorbance per minute (ΔA).

The molar absorptivity (ϵ) of Δ^4-cholestenone was 12.2×10^3 liter $mole^{-1}$ cm^{-1} when it was solubilized in the above manner.

Cholesterol oxidase activity may therefore be calculated as:

$$\frac{(\Delta A \times \text{reaction volume} \times 0.082)}{\text{volume of enzyme taken}} = 5.1 \times \Delta A \text{ units/ml}$$

General Properties of the Enzyme

Stability. Cholesterol oxidase prepared by use of ion-exchange chromatography is very stable. No detectable change in activity occurred when the enzyme was stored in buffered Triton X-100 for six months at 4 °C. The enzyme is also highly heat stable, which allows it to be used analytically at temperatures as high as 50 °C. It is active in aqueous ethanolic solutions containing as much as 300 ml of ethanol per liter, and in buffers ranging from pH 4.0 to pH 9.0; its activity was maximal at pH 7.0. The Michaelis constant with the assay conditions described was found to be 1.4×10^{-5} mol/liter.

Specificity. The specificity of the enzyme was investigated by comparing the rates of oxidation of cholesterol analogs with that of cholesterol at the same molarity, assuming that the Δ^4-3-oxo products would have the same molar absorptivities as does Δ^4-cholestenone. From the data in Table 2, it appears that the enzyme is specific for 3β-stero'ls and requires a double bond in the Δ^5- or Δ^4-positions. Shortening the cholesterol side chain markedly diminishes the affinity of the enzyme for these substrates. The concentrations of potentially interfering physiological sterols in serum are therefore less than 0.001 that of cholesterol.

Table 2. Substrate Specificity of Cholesterol Oxidase[a]

Substrate	Rate of enzymic oxidation[b]
4-Cholesten-3β-ol	77
3β-Hydroxy-5-cholene-24-oic acid	23
5-Pregnen-3β-ol	6.3
Androst-5-ene-3β-ol	0.9
5α-Cholestan-3β-ol	0
5β-Cholestan-3β-ol	0

[a] Rates of enzymic oxidation were measured by substituting the sterols for cholesterol in the assay of cholesterol oxidase. It was assumed that Δ^4-3-oxo product would have the same molar absorptivity as Δ^4-cholestenone.

[b] The rate of enzymic oxidation is expressed as a percentage of the rate of oxidation of cholesterol at the same molarity.

These properties make the enzyme very suitable as an analytical reagent. Several approaches to the assay of cholesterol in serum are therefore possible:

- measurement of oxygen consumption with an oxygen electrode
- measurement of hydrogen peroxide production, enzymatically or otherwise
- measurement of Δ^4-cholestenone, either directly or as a derivative.

We prefer to measure hydrogen peroxide production because this approach is most likely to produce an easily automated colorimetric assay. The color reaction of choice is the direct formation of a chelate complex of hydrogen peroxide with quadrivalent titanium and xylenol orange (*15*), because this provides an inexpensive, one-enzyme system with very stable reagents and a fast, specific, and stable color reaction.

Assay of Total Cholesterol in Serum

Principle. Cholesterol is released from lipoprotein complexes and hydrolyzed from its esters by alkali. After neutralization, the hydrolysate is treated with mercuric ions to remove interfering reducing substances generated in the saponification step. The liberated cholesterol, held in solution by Triton X-100, is subsequently oxidized to Δ^4-cholestenone with the simultaneous production of hydrogen peroxide, which is chelated with xylenol orange and quadrivalent titanium. The absorption of the complex is measured at 550 nm vs. a reagent blank in which a solution of bovine serum albumin in water (70 g/liter) replaces serum.

Optimization of procedure. In the saponification step, a 1.0 molar concentration of alkali is required, to keep protein in solution. All the cholesterol esters are hydrolyzed by incubation for 5 min at 75 °C, as shown by thin-layer chromatography. With most sera a light precipitate forms, but this redissolves completely at the neutralization stage. Although the ethanolic alkali contains 50 ml of Triton X-100 per liter, this is subsequently diluted to 3.5 ml/liter; at

this concentration, protein and cholesterol are maintained in solution and the reaction is not inhibited.

In neutralizing the hydrolysate, the pH must be maintained above 7.5 to prevent formation of a precipitate when mercuric chloride is added. It is thus necessary to buffer the neutralizing reagent, but buffer concentration must be kept low to prevent formation of a precipitate when the titanium–xylenol orange reagent is subsequently added. Tris(hydroxymethyl)aminomethane phosphate, which was included in the neutralizing reagent, was found to maintain the pH satisfactorily between pH 7.5 and 7.8.

After saponification and neutralization of serum or "Serachol" reference serum (William R. Warner Ltd, Eastleigh, England) samples, reducing substances were demonstrably present equivalent to approximately 0.4 μmol of hydrogen peroxide per milliliter. These substances were not formed when reagent blanks or cholesterol standards in isopropanol were substituted for serum, and it was necessary to remove them before enzymatic oxidation in order to prevent underestimation of total cholesterol in serum. They were removed by adding excess hydrogen peroxide after neutralizing the saponification mixture. Residual peroxide was then destroyed by brief incubation with catalase, which was in turn inhibited with sodium azide before effecting the enzymatic oxidation of cholesterol. We expected that this procedure would overcome potential interference and have the added advantage that oxygen generated by the action of catalase on excess peroxide might activate the cholesterol oxidase system. In practice, reproducibility proved to be poor, and further work showed that interfering reducing substances could be removed more effectively by oxidation with mercuric chloride. Mercuric ions strongly inhibit cholesterol oxidase; this effect may be removed by adding sodium azide, which also serves as a catalase inhibitor and probably is effective because mercuric azide is formed; this cannot inhibit the enzyme. Figure 3 indicates the concentration of mercuric ions required for complete removal of interfering reducing substances, and hence maximal color development, when Serachol samples were taken through the assay procedure.

Figure 4 demonstrates the pH dependence of the color reaction. The composition of the combined color reagent has been selected to produce a final pH of 1.15 which gives adequate sensitivity without creating the solubility problems that arise at higher pH values. The color reaction has been shown to be linear up to 20 mmol of cholesterol per liter.

With the conditions specified, 0.25 U of cholesterol oxidase in a reaction volume of 1.25 ml is sufficient to effect complete oxidation, within 5 min, of samples containing as much as 20 mmol of cholesterol per liter (773 mg/dl).

Reagents

Ethanolic KOH, 1 mol/liter. Triton X-100 (50 ml) is mixed with about 600 ml of ethanol (reagent grade). A "Volucon" ampoule (May & Baker Ltd., Dagenham, England), which contains enough KOH to make 1.0 liter of 1 mol/liter KOH, is added and the mixture diluted to 1.0 liter with more ethanol.

Tris(hydroxymethyl)aminomethane phosphate buffer, pH 7.6. Tris-(hydroxymethyl)aminomethane (10 mmol/liter) is titrated with potassium dihydrogen orthophosphate (20 mmol/liter) to give a solution of pH 7.6.

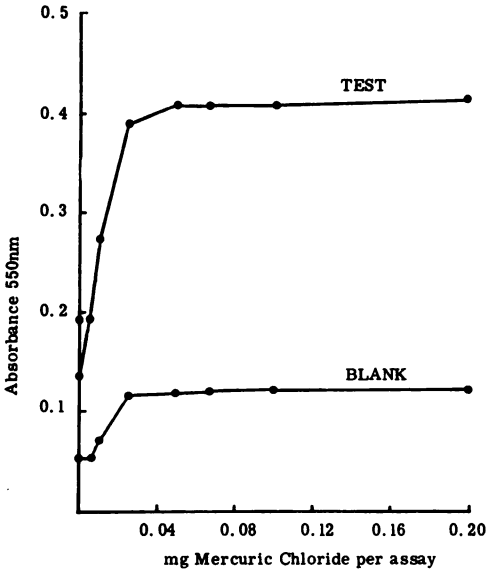

Fig. 3. Removal of interfering reducing substances generated during saponification of serum by oxidation with mercuric chloride

A "Serachol" sample, containing 9.0 mmol of cholesterol per liter, was saponified. Portions were then assayed for cholesterol by the enzymatic technique by using various concentrations of mercuric chloride in the oxidizing reagent. Maximum color developed when at least 50 μg of mercuric chloride was used per test, corresponding to complete oxidation of interfering reducing substances

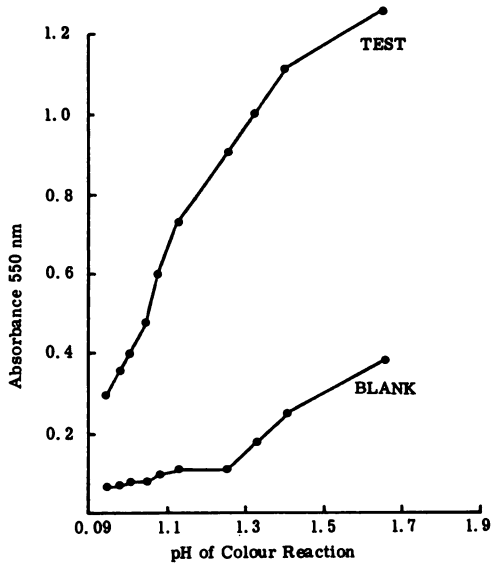

Fig. 4. pH-dependence of color development by the titanium/xylenol orange/hydrogen peroxide chelate

The assay procedure was followed, using pooled serum, with the following variations: 100 μl of hydrogen peroxide (3.5 mmol/l in 0.1 mol/l phosphate buffer pH 7.0 containing Triton X-100, 15 ml/liter) was added in place of enzyme. The color reaction was done with combined color reagent containing varying concentrations of acid to vary the final pH. In the blanks, hydrogen peroxide was omitted from the buffered Triton X-100 solutions

Neutralizing reagent (A). Hydrochloric acid (1 mol/liter), 16.6 ml, is diluted to 100 ml with tris(hydroxymethyl)aminomethane phosphate buffer (pH 7.60). After the preparation of each new batch of the reagent, the pH after neutralization of hydrolysate in the assay procedure should be checked; it should be between pH 7.5 and 7.8 at 37 °C.

Oxidizing reagent (B). Mercuric chloride (14 mg, or 0.2 ml of a saturated aqueous solution) and sodium azide (1.0 g) are dissolved in water and diluted to 100 ml.

Cholesterol oxidase solution. This should contain from 1 to 5 U of cholesterol oxidase per milliliter in phosphate buffer (0.1 mol/liter, pH 7.6) with 20 ml of added Triton X-100 per liter; 0.25 U (50 μl at 5 U/ml) of enzyme are used for each test.

Stock titanium solution. Dissolve titanium dioxide (0.80 g) and ammonium sulfate (5.0 g) in 100 ml of concentrated sulfuric acid by cautious heating. The temperature should not be allowed to exceed 230 °C, to avoid decomposition of ammonium sulfate.

Stock xylenol orange. Dissolve xylenol orange (0.76 g) in 1.0 liter of an aqueous solution of Triton X-100 (20 ml/liter).

Combined color reagent (C). Add 2.5 ml of stock titanium solution and 3.0 ml of concentrated sulfuric acid to about 200 ml of distilled water and dilute to 250 ml with more water. Add to this titanium solution, an equal volume of the stock xylenol orange solution with stirring. It is most important to add the xylenol orange to the acid titanium solution to prevent formation of a complex that can occur at a higher pH.

Standards. Cholesterol solutions in isopropanol, prepared from cholesterol biochemical standard (BDH Chemicals Ltd., Poole, England), were used as primary standards. Working standards were prepared from Serachol which was assayed and then diluted appropriately with a solution (7.0 g/dl) of bovine serum albumin (Armour Co., Eastbourne, Eng.) in physiological saline (9 g of NaCl per liter).

Procedure

Add 200 μl of serum, standard, or blank to 1.0 ml of ethanolic KOH, mix thoroughly, and keep in stoppered tubes for 5 min at 75 °C. Cool rapidly to room temperature and add 100 μl of the mixture to 500 μl of the neutralizing agent. Mix, and add 500 μl of oxidizing reagent B. Add 50 μl of the cholesterol oxidase solution, mix, and keep at 37 °C for 5 min. Finally add 1.0 ml of color reagent C and let stand for a further 5 min at 37 °C.

The absorbance of the test or standard is read at 550 nm vs. the reagent blank.

When blank determinations were carried out on 12 different sera, by substituting 0.1 molar phosphate buffer containing 15 ml of Triton X-100 per liter for enzyme in the assay procedure, a mean blank absorbance of 0.098 (CV, 4.3%) was obtained. We consider this variation, of a few milliabsorbance units, shows that test blanks are unnecessary. Serachol and bovine serum albumin (7.0 g/dl), treated similarly, gave the same mean blank value as sera, but water or cholesterol standards in isopropanol gave slightly lower blank values. It seems reasonable therefore that Serachol diluted in bovine serum albumin can be used for calibration while using bovine serum albumin as a single reagent blank for both standards and tests. Primary cholesterol standards in isopropanol can be used when the appropriate blank is taken through the procedure.

Evaluation of the Assay

Precision. Within-batch precision for the assay was calculated from the results for 12 consecutive assays of Serochol stated (labeled value) to contain 346 mg/dl (8.95 mmol/liter). A mean value of 8.90 mmol/liter (SD, ±0.26; CV, 2.9%) was obtained.

Inter-method comparison. Twenty-nine sera, from randomly selected hospital patients, were assayed enzymically and by an automated version (*16*) of the Abell technique involving saponification, extraction, and Liebermann-Burchard reaction. The correlation obtained is shown in Figure 5. The negative intercept on the *y* axis suggests that the enzymatic technique is more specific than is the automated Abell assay. Similar findings have been reported for gas-chromatographic methods of assay (*17, 18*), which have also been claimed to be more specific than colorimetric techniques. However, sufficient data are not yet available to make detailed comparisons of the relative specificities of colorimetric, enzymatic, and gas-chromatographic methods.

The application of the present method in the routine laboratory and participation in a rigorous quality control program will be necessary to fully evaluate its performance.

Interferences. Tammes and Nordschow (*15*) have discussed possible interferences from certain anions, cations, and anticoagulants in the use of the quadrivalent titanium/xylenol orange reaction to measure

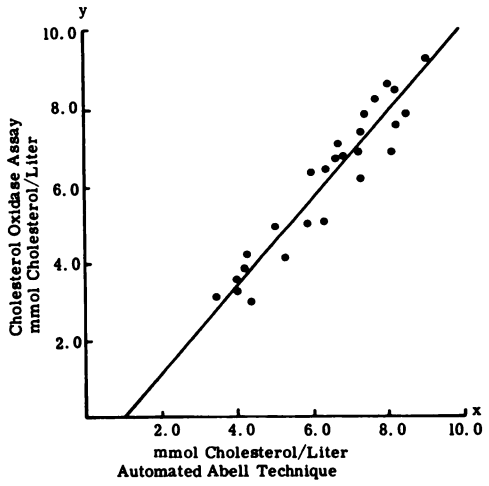

Fig. 5. Correlation between results for serum total cholesterol, as measured by the enzymatic assay (*y* axis) and by the automated Abell technique (*x* axis)
n = 29, slope = 1.13, intercept = −1.07, correlation coefficient (*r*) = 0.958

Table 3. Effect of Added Hemoglobin and Bilirubin on the Enzymatic Assay of Cholesterol in Samples of a Pooled Plasma[a]

Bilirubin added, mg/dl	Cholesterol found, mmol/liter	Hemoglobin added, mg/dl	Cholesterol found, mmol/liter
0	3.9	0	4.0[b]
5	4.1	40	3.9
10	4.2	80	3.9
15	3.9	120	3.7
20	4.1	160	3.8
25	4.1	200	3.9

[a] Bilirubin and hemoglobin were added to different aliquots of the same plasma pool to give the concentrations indicated.
[b] 145 mg/dl.

hydrogen peroxide and have shown that these are not encountered when serum is used as the sample.

Although cholesterol oxidase can be prepared catalase free, the presence of azide in the assay system safeguards against catalase interference arising from this source or from bacterial contamination of reagents.

To investigate the effects of hemoglobin and bilirubin on the enzymic assay, we prepared a fresh pool of plasma containing 0.5 mg of bilirubin per deciliter and no detectable hemoglobin. Bilirubin dissolved in dimethyl sulfoxide (*19*) was added to a portion of the pool to give a stock solution containing 25 mg of bilirubin per deciliter. This stock was then diluted with plasma, to which an equivalent amount of dimethyl sulfoxide had been added, to give solutions ranging from 0.5 to 25 mg of bilirubin/dl.

Plasma specimens with hemoglobin concentrations ranging from zero to 200 mg/dl were prepared from a hemolysate of erythrocytes. The plasma diluent was prepared by adding an equivalent amount of physiological saline to a portion of the pool.

The plasma samples containing added bilirubin or hemoglobin were then assayed for cholesterol by our technique, with the plasma diluent in each case serving as a baseline value. The results (Table 3) show that bilirubin and hemoglobin have no effect on the results of the assay, even at the highest concentrations examined.

Discussion

The present findings strongly suggest that the enzymatic method described here would be more specific and precise in routine use than are conventional techniques. Moreover, the assay system does not contain highly corrosive reagents—a distinct advantage for automation. Hydrogen peroxide chelation, however, is only one of the many possible approaches to the assay of serum cholesterol by use of this robust enzyme. Peroxidase/oxygen acceptor systems are under investigation and have been shown to be compatible with the above reaction conditions. Automation of the assay with both continuous and discrete systems is also under development.

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