the hydrobromide of IIIc (876 mg). This was dissolved in H_2O (20 ml) and passed through a column which was packed with Amberlite IRA-402 (HCO_3^-) (8 ml). The column was further eluted with H_2O (70 ml). The eluate was concentrated to a small volume, providing IIIc (603 mg, 69%). Recrystallization from 70% (v/v) aq. ethanol gave colorless needles, mp 214—216° (decomp.) (lit.[6c] mp 227—228°)[12]; UV $\lambda_{max}^{95\% EtOH}$ 228 nm (ε 20700), 273 (11800); $\lambda_{max}^{H_2O}$ (pH 1) 260 (12200); $\lambda_{max}^{H_2O}$ (pH 7) 267 (11300); $\lambda_{max}^{H_2O}$ (pH 13) 272 (14500). *Anal.* Calcd. for $C_8H_9N_5$: C, 54.84; H, 5.18; N, 39.98. Found: C, 54.62; H, 5.23; N, 40.08.

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Determination of Total and Free Cholesterol by using Cholesterol Oxidase from *Streptomyces*

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Total and free serum cholesterol was determined by using a new cholesterol oxidase preparation obtained from *Streptomyces*. Cholesterol esters were hydrolyzed by cholesterol esterase, and free cholesterol was oxidized to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide, which oxidatively coupled with 4-aminoantipyrine and phenol to form the quinoneimine dye. The produced color was measured by its absorption at 500 nm.

The results of serum cholesterol determination correlate well with those obtained by Zak-Henly and Kiliani method. Moreover, this enzymatic method is simpler and more sensitive than the conventional method.

Keywords——total cholesterol determination; free cholesterol determination; cholesterol oxidase; cholesterol esterase; quinoneimine dye

Recently, the assay method of serum cholesterol using cholesterol dehydrogenase isolated from *Nocardia* has been reported by Flegg.[2)] Afterward, Allain, *et al.*[3)] and Masamichi, *et al.*[4)] reported the procedures using cholesterol esterase, cholesterol oxidase and peroxidase to determine the total serum cholesterol.

We devised a new method to screen anticholesterol substances produced by microbes on a basis of the phenomenon that polyene antibiotics lost antiyeast activity by cholesterol.[5)] In the course of screening, an active substance was found in the culture filtrate of *Streptomyces violascens*. This active substance was identified as a new cholesterol oxidase and purified.[5,6)] Through a study of *Streptomyces* cholesterol oxidase, we investigated the possibility to determine serum cholesterol by using our enzyme, and found that total and free serum cholesterol could be estimated in a similar manner to the reported enzymatic method.

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Materials and Methods

Cholesterol oxidase (EC 1.1.3.6.) was prepared from the culture filtrate of *Streptomyces violascens*, as previously described.[6] Serachol (total cholesterol, 390 mg/dl, free cholesterol, 97 mg/dl) was purchased from Ono Pharmaceutical Co. Cholesterol esterase (EC 3.1.1.13) (from beef pancreas) was from Miles Laboratories. All other chemicals were obtained from commercial sources and were of analytical grade.

The activity of *Streptomyces* cholesterol oxidase was measured by the two different methods. In one method, the activity of the enzyme was measured by the method of Stadtman, *et al.*[7,8] which was determined cholest-4-en-3-one at 240 nm after the reaction. One unit of cholesterol oxidase was defined as the amount of enzyme oxidizing 1 μmole of cholesterol to cholest-4-en-3-one per min at 30°. The other method in order to select detergent, was based on the formation of quinoneimine dye. Serachol (100 μl) was added to 2.5 ml of 0.1M phosphate buffer (pH 6.8) containing 0.82 mM 4-aminoantipyrine, 14 mM phenol, 4.75 units of peroxidase and cholesterol oxidase. The solution was mixed and incubated at 37° for 30 min. The produced quinoneimine dye was determined at 500 nm.

The activity of cholesterol esterase was measured by the method of Allain, *et al.*[3] Serachol (25 μl) was added to 2.5 ml of the incubation mixture for cholesterol oxidase assay (quinoneimine dye method) containing 0.0925 unit of cholesterol oxidase and cholesterol esterase. The solution was mixed and incubated at 37° for 30 min. The produced quinoneimine dye was determined at 500 nm. One uint of cholesterol esterase was defined as the amount of enzyme hydrolyzing 1 μmole of cholesterol ester to free cholesterol per min at 37° in the incubation mixture containing 20 mM sodium cholate.

TABLE I. Effect of the Detergents on the Enzyme Activity of Cholesterol Oxidase and Esterase

Detergent	Cholesterol oxidase		Cholesterol esterase	
	Optimum concn.	Relative activity	Optimum concn.	Relative activity
None		100		0
Sodium cholate	8 mM	292	4—8 mM	100
Sodium deoxycholate	2—8 mM	308	1 mM	17
Sodium taurocholate	1 mM	194		—
Sodium tauroglycocholate	2 mM	294		—
Triton X-100	0.4%	312	0.4%	13
Tween 20	>0.02%	inhibitory		—
Emulgen 106	0.025%	184		—
Emulgen 109P	0.4%	300	0.4%	10
Emulgen 120	0.2%	180		—

Cholesterol oxidase was measured by the method based on the formation of quinoneimine dye.

TABLE II. Optimum Condition for Cholesterol Determination

Ingredient	Concentration (mM)	
	Free cholesterol	Total cholesterol
4-Aminoantipyrine	0.82	0.82
Phenol	14	14
Sodium cholate	20	20
Cholesterol esterase		400 U/l
Cholesterol oxidase	37 U/l	37 U/l
Peroxidase	1900 U/l	1900 U/l
Sample	0.1 ml	0.025 ml
	in 0.1 M sodium phosphate buffer (pH 6.8)	

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Optimization studies were performed with Serachol for each of the components contained in the assay mixture. The coloring reaction was based on the formation of quinoneimine dye.[9] In the assay of total cholesterol, serum (25 μl) was incubated with 2.5 ml of the assay mixture containing, 0.1M phosphate buffer (pH 6.8), 0.82 mM 4-aminoantipyrine, 14 mM phenol, 20 mM sodium cholate, 1 unit of cholesterol esterase, 0.0925 unit of cholesterol oxidase and 4.75 units of peroxidase for 30 min at 37° and the absorbance of produced quinoneimine dye was determined at 500 nm against the blank test. In the assay of free cholesterol, serum (100 μl) was added in the above mixture excluding cholesterol esterase.

Total and free cholesterol concentrations of unknown samples were determined from a standard calibration curve constructed by using Serachol. Serachol was diluted with 7% bovine serum albumin to standardize the effect of protein.

Results and Discussion

As the detergents were essential to increase the activity of *Streptomyces* cholesterol oxidase and cholesterol esterase, the effects of various detergents on the activity of these enzymes were investigated. As shown in Table I, sodium cholate was the most effective on these enzyme activities. Triton X-100 and Emulgen 109 P activated *Streptomyces* cholesterol oxidase equally to sodium cholate, but these detergents did not activate cholesterol esterase as sodium cholate. Therefore, we used sodium cholate as the detergent in this assay method.

Optimization studies were performed for the several components *e.g.* cholesterol esterase and *Streptomyces* cholesterol oxidase and the final optimized condition was summarized in Table II.

Under the conditions in Table II, cholesterol esters in Serachol were completely hydrolyzed to free cholesterol by cholesterol esterase, and free cholesterol was completely oxidized to cholest-4-en-3-one by *Streptomyces* cholesterol oxidase. These enzymatic reaction products were confirmed by thin layer chromatography.

The reaction was accomplished within 30 min at 37°, and the produced color was stable at least for 60 min at room temperature.

In the chemical method, some biological materials such as bilirubin were reported to interfer the quantitative determination of serum cholesterol.[10] So, the effects of anticoagurants and biological materials to determine cholesterol were studied in the present method. As shown in Table III, bilirubin and ascorbic acid interfer little the determination of cholesterol. However, the effect of bilirubin was less than that obtained by the chemical method.

Since hydrogen peroxide is produced by cholesterol oxidase in the present method, it is considered that catalase in the serum may affect the colorization. However, as shown

Table III. The Effects of Anticoagulants and Biological Materials on Cholesterol Determination

Compound	Concn.	Cholesterol (mg/dl)		Difference (%)	
		Free	Total	Free	Total
None		97	390		
Heparin	100 U/ml	98	396	+1.0	+1.5
Citric acid	10 mg/ml	97	389	±0	−0.3
EDTA	10 mg/ml	97	387	±0	−0.8
Creatinine	10 mg/dl	100	390	+3.1	±0
Ascorbic acid	4 mg/dl	89	381	−8.2	−2.3
Uric acid	10 mg/dl	99	387	+2.1	−0.8
Glucose	600 mg/dl	98	390	+1.0	±0
Bilirubin	10 mg/dl	101	391	+4.1	+0.3
Glutathione	5 mg/dl	92	385	−5.2	−1.3
Hemoglobin	75 mg/dl	103	394	+6.2	+1.0
Catalase	100 U/ml	96	390	±0	±0

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Table IV. Relative Rates of Substrate Specificity of Present Method

Sterol	Relative colorization	Sterol	Relative colorization
Cholesterol	100	5-Cholesten-3β,20α-diol	100
5α-Cholestan-3β-ol	90	Ergosterol	42
7-Dehydrocholesterol	49		

in Table III, the colorization of the present method was not affected in the indicated amount of catalase.

Tomioka, *et al.*[6] demonstrated that *Streptomyces* cholesterol oxidase oxidized preferentially 3β-sterols and Δ^5-sterols. Richmond[11] showed that *Nocardia* cholesterol oxidase was specific for 3β-sterols and required a double bond in the Δ^5 or Δ^4 position of the sterol.

We also assessed the substrate specificity of the present method by incubating at 37° for 30 min with isopropanol solution of several sterols (250 nmoles). The results were shown in Table IV. The *Nocardia* cholesterol oxidase oxidized 5α-cholestan-3β-ol and 5-cholesten-3β, 20α-diol more slowly than the *Streptomyces* enzyme. However, with 7-dehydrocholesterol which is the most abundant serum sterol except cholesterol,[12] the present method gave less error than the method using *Nocardia* enzyme.

The results obtained by the present method were compared with those obtained by the chemical ones. The total cholesterol in human sera were assayed by the present method and the method of Zak-Henly.[13] The calculated correlation coefficient was 0.985 and the linear regression equation was $Y= 1.10X$-14.0. The free cholesterol in human sera were assayed by the present method and the method of Kiliani.[14] Its coefficient was 0.995 and its equation was $Y= 0.782X+9.46$. The calculated coefficient and the linear regression equation obtained between the present method and the chemical methods indicate excellent agreement.

The ester ratio of serum cholesterol can be easily determined by the present method. In the future, this ester ratio may be usefull in clinical practice. Furthermore, this method may be easily adapted for automated procedure because of avoiding corrosive mineral acids.

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