CLIN. CHEM. 20/4, 470–475 (1974)

Enzymatic Determination of Total Serum Cholesterol

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An enzymatic method is described for determination of total serum cholesterol by use of a single aqueous reagent. The method requires no prior treatment of sample and the calibration curve is linear to 600 mg/dl. Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase (EC 3.1.1.13). The free cholesterol produced is oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm. The method is reproducible, and the results correlate well with those obtained by automated Liebermann–Burchard procedures (AA-2 and SMA 12/60) and the method of Abell et al. The present method affords better specificity than those previously reported and has excellent precision.

The literature is replete with methods for determination of serum cholesterol, and major reviews have described the interferences and sources of variation to which these methods are prone (*1–5*). Interest in microbiological enzymes capable of degrading cholesterol dates back many years (*6–8*), and two workers have recently shown that certain enzymes may be applied to the assay of cholesterol. Flegg (*9*) used "cholesterol dehydrogenase" isolated from *Nocardia erythropolis* (*6*) for the assay of total cholesterol, by incubating saponified serum extracts with the enzyme for as long as 2 h and measuring the Δ^4-cholestenone produced by its absorption at 240 nm. Richmond isolated a cholesterol:oxygen oxidoreductase from another species of *Nocardia* and applied the purified enzyme to the direct assay of cholesterol in saponified serum by measuring the hydrogen peroxide produced (*10, 11*).

The above procedures are based on the nonenzymatic hydrolysis of cholesterol esters before measurement of free cholesterol.

Hernandez and Chaikoff (*12*) and later Hyun et al. (*13*) isolated cholesterol ester hydrolase (EC 3.1.1.13) from pork pancreas and rat pancreatic juice, respectively, which was effective in converting cholesterol esters to free cholesterol.

This report describes a procedure involving the use of three enzymes for determination of total serum cholesterol; the hydrogen peroxide generated by cholesterol oxidase is measured by the oxidative coupling of 4-aminoantipyrine and phenol (*14*). The sequence of reactions is shown in Figure 1. The data presented suggest that the method is preferable to older methods in terms of specificity, dynamic range, and simplicity.

Materials and Methods

Reagents

Cholesterol ester hydrolase (EC 3.1.1.13) was obtained from Ames Laboratories, a Division of Miles Laboratories, Inc., Elkhart, Ind. 46514, and had an activity of 0.018 U/mg. Cholesterol oxidase[4] from *Nocardia* as isolated and characterized by Richmond (*10*), was obtained from Whatman Biochemicals, Ltd., Springfield Mill, Maidstone, Kent, England ME142LE, and had an activity of 5.0 U/ml. The peroxidase was from Worthington Biochemical Corp., Freehold, N. J. 07728, and had an activity of 830 U/mg.

Other materials were obtained as follows: sodium cholate (General Biochemicals, Chagrin Falls, Ohio 44022); phenol (Mallinckrodt Chemical Works, St. Louis, Mo. 63160); 4-aminoantipyrine (J. T. Baker Chemical Co., Phillipsburg, N. J. 08865); Triton X-100 (Rohm and Hass, Philadelphia, Pa. 19105); ash-free cholesterol (Pfanstiehl, Waukegan, Ill. 60085); aqueous cholesterol standard (TEKIT: Searle Diagonostic, Inc., Columbus, Ohio 43216); Metrix normal serum control (Armour Pharmaceutical Co., Chicago, Ill. 60690); Serachol (General Diagnostics Div., Warner-Lambert Co., Morris Plains, N. J. 07950); Silica Gel F_{254} pre-coated plates 5 × 20

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Received Jan. 8, 1974; accepted Jan. 31, 1974.

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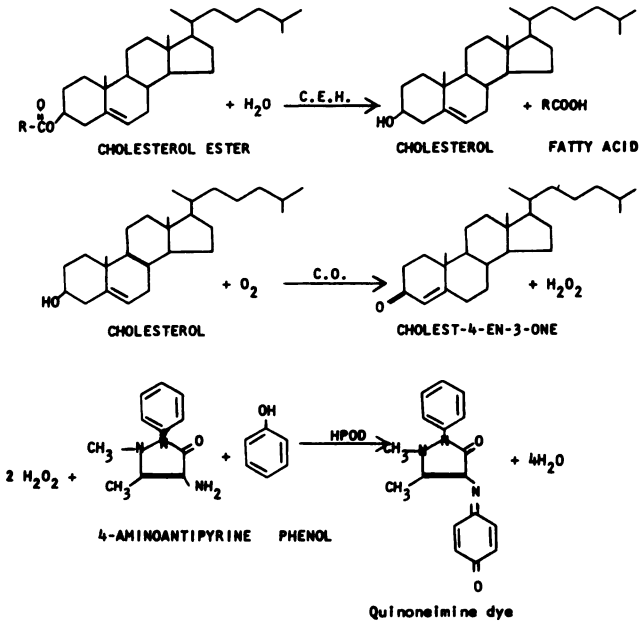

Fig. 1. Reaction scheme for the enzymatic measurement of total serum cholesterol

cm, 0.25 cm thick (Brinkmann Instruments, Inc., Westbury, Long Island, N. Y.); Carbowax 6000, 5α-cholestan-3β-ol, 7-dehydrocholesterol, 5-cholesten-3β-20α-diol, 7-cholesten-3β-ol, and ergosterol were from Schwarz/Mann Research Laboratories, Orangeburg, N. Y. 10962.

Enzymatic Assays

Cholesterol oxidase. The assay of cholesterol oxidase (C.O.)[5] is based on the conversion of cholesterol to cholest-4-en-3-one, which has an absorption maximum at 240 nm owing to the conjugated carbonyl group in ring A (*10*). To 3.0 ml of phosphate buffer (0.10 mol/liter, pH 7.0, and containing 0.5 ml of Triton X-100 per liter) in a 1.0-cm cuvette, add 50 μl of 300 mg/100 ml cholesterol solution (Tekit, aqueous cholesterol standard) and mix by inversion. Zero the spectrophotometer against a blank of the same buffer. Add 10 μl of an aqueous solution of cholesterol oxidase containing about 2 U/ml, and measure the rate of change in absorbance at 240 nm at 37 °C. One enzyme unit is defined as that amount of cholesterol oxidase that will promote the conversion of 1 μmol of cholesterol to cholest-4-en-3-one per minute under the prescribed test conditions. The millimolar absorptivity for cholest-4-en-3-one used here is 18.0 liter $mmol^{-1}$ cm^{-1}, as reported by Bladon (*15*).

Cholesterol ester hydrolase. The assay of cholesterol ester hydrolase (C.E.H.) is based on the formation of a quinoneimine dye in the sequence of reactions shown in Figure 1. With substrate, C.O., and HPOD in excess, the rate of decomposition of cholesterol esters is determined by measuring the rate of color development at 500 nm.

[5] Nonstandard abbreviations used: C.E.H., cholesterol ester hydrolase (EC 3.1.1.13); C.O., cholesterol oxidase (cholesterol:oxygen oxidoreductase, EC 1.1.3.6); HPOD (horseradish source; donor:H_2O_2 oxidoreductase, EC 1.11.1.7).

Add 50 μl of Serachol to 3.0 ml of a phosphate buffer (0.10 mol/liter, pH 6.7) containing, per liter, 3.0 mmol of sodium cholate, 0.8 mmol of 4-aminoantipyrine, 14 mmol of phenol, 67 000 U of HPOD, 120 U of cholesterol oxidase, and 0.17 mmol of Carbowax-6000. Mix the solution and incubate in a 1.0-cm cuvette at 37 °C for 5 min. Add 0.10 ml of a solution of C.E.H. in phosphate buffer (pH 6.7; 0.10 mol/liter) containing about 0.05 U/ml and measure the rate of change in absorbance at 500 nm at 37 °C.

One unit of cholesterol ester hydrolase activity is that amount of enzyme decomposing 1 μmol of cholesterol ester per minute at 37 °C. The millimolar absorptivity for the dye formed under these conditions was 5.33.

Instrumentation

A Perkin-Elmer Model 46 spectrophotometer, thermostatted at 37 °C, was used to assay all enzymes. We used the Abbott Bichromatic Analyzer-100 (ABA-100), with a 500/600-nm filter set and a 1:101 (sample:total volume) syringe plate in the end point mode at 37 °C for optimization and performance studies.

Procedure

Optimization studies were performed for each of the components of the cholesterol assay. A native human serum pool with a total cholesterol content of 500 mg/dl was used in the optimization of cholesterol ester hydrolase, sodium cholate, and pH; a 600 mg/dl cholesterol standard in isopropanol was used in the remaining optimization studies. When we had ascertained the optimum concentration of a particular ingredient it was maintained at that concentration while the next ingredient was optimized.

In the manual assay of total cholesterol by the present method, 30 μl of serum is incubated with 3.0 ml of reagent for 10 min at 37 °C and the absorbance at 500 nm is measured vs. a reagent blank. Concentrations of unknown samples are determined from a standard curve constructed by using cholesterol standards in isopropanol.

Results

Optimization curves for several of the ingredients are shown in Figures 2–6, and the final optimized formula is shown in Table 1. Carbowax-6000 is included in the formulation to aid in maintaining free cholesterol in solution. The sodium cholate is required for hydrolysis of cholesterol esters by cholesterol ester hydrolase. The reaction is complete in 10 min at 37 °C and the final color produced is stable for at least 90 min (Figure 7). The linearity of the method is shown in Figure 8.

Thin-Layer Chromatography

In order to visually study the effects of the cholesterol ester hydrolase and cholesterol oxidase, thin-layer chromatography was performed with pre-coat-

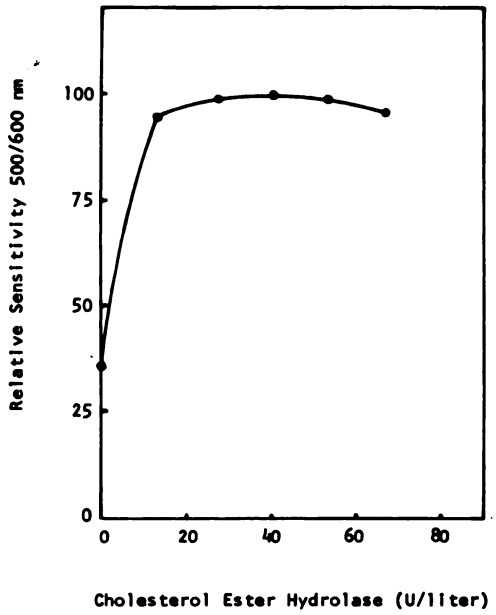

Fig. 2. Effect of cholesterol ester hydrolase concentration on final absorbance

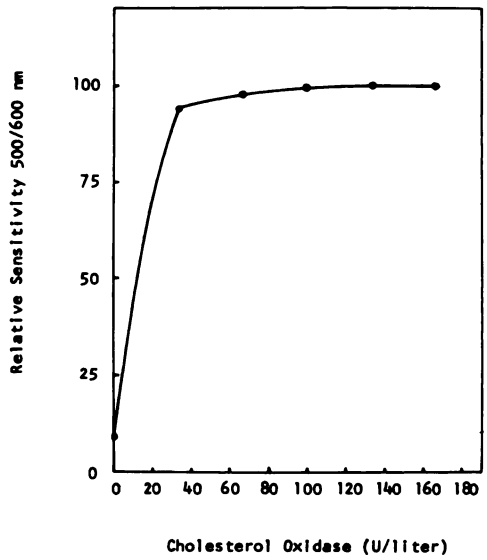

Fig. 3. Effect of cholesterol oxidase concentration on final absorbance

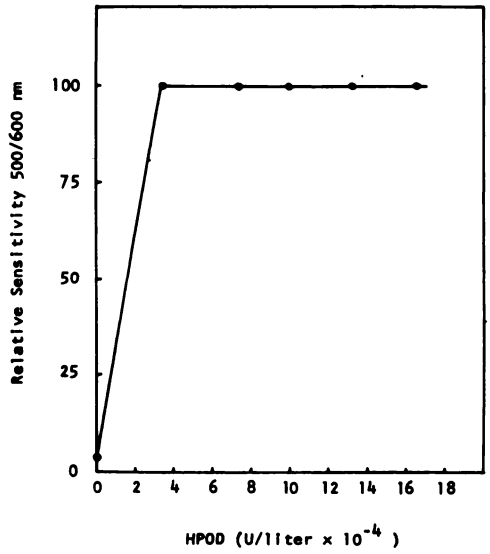

Fig. 4. Effect of horseradish peroxidase concentration on final absorbance

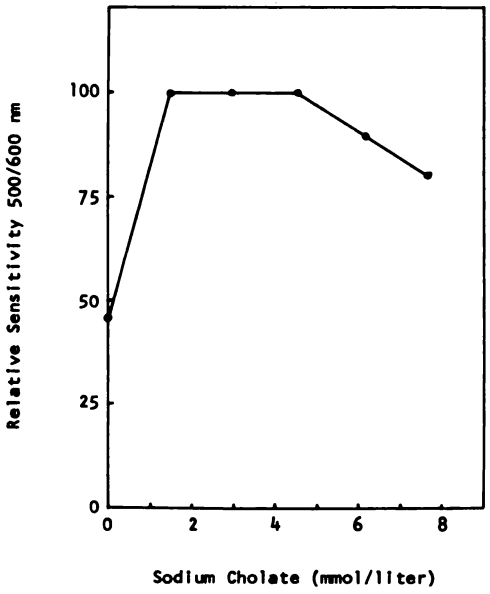

Fig. 5. Effect of sodium cholate concentration on final absorbance

Table 1. Optimized Formula for Present Method

Ingredient	Concentration, mmol/liter
Sodium cholate	3
4-Aminoantipyrine	0.82
Phenol	14
Na_2HPO_4	50
NaH_2PO_4	50
Carbowax-6000	0.17
Cholesterol ester hydrolase	33 U/liter
Cholesterol oxidase	117 U/liter
Peroxidase	67000 U/liter
$pH_{25\,°C}$ 6.70 ± 0.10	

ed Silica Gel F_{254} plates. Lipids were separated with the solvent system hexane:diethyl ether:acetic acid (80:20:1.5, by vol) and spots were made visible by placing the plates into an atmosphere of iodine vapors.

Native human serum (30 µl) was added to 3.0 ml of reagent (Table 1) or to similar solutions omitting the hydrolase and oxidase, and allowed to incubate at 37 °C for exactly 10 min, after which time 5 ml of chloroform:methanol (2:1, by vol) was added and the mixture shaken vigorously and centrifuged 5 min at 5000 rpm. The chloroform layer was separated and evaporated under a stream of air, and the residue was dissolved in 150 µl of hexane. The hexane solution was then spotted on the silica-gel plates and al-

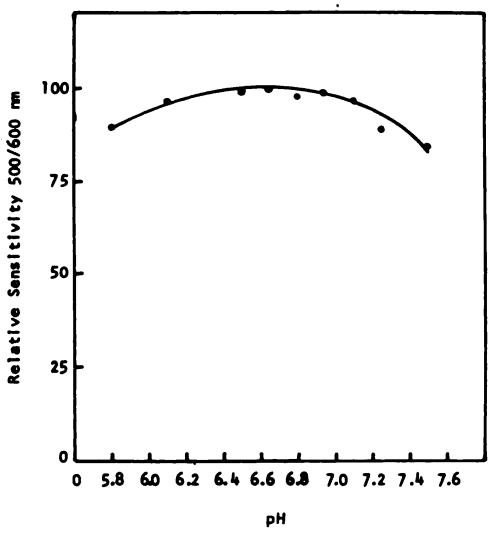

Fig. 6. Effect of pH on final absorbance

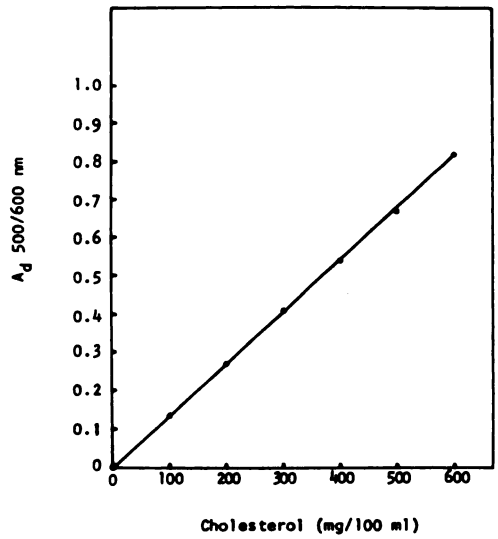

Fig. 8. Proportionality of absorbance to cholesterol concentration

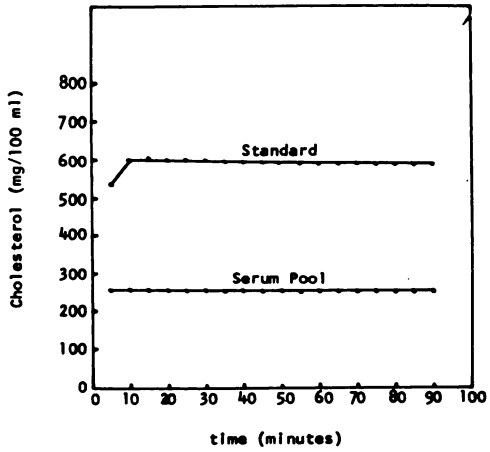

Fig. 7. Stability of final color produced

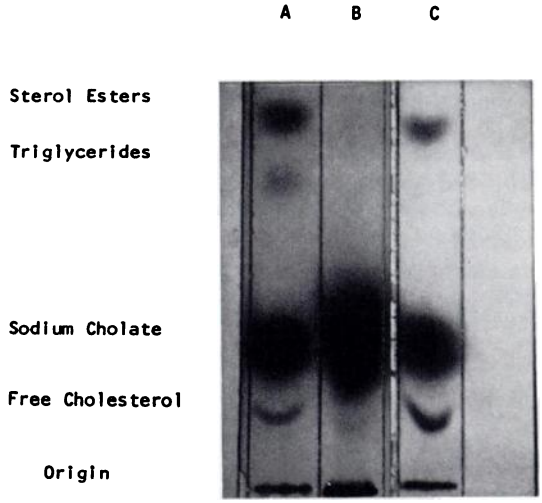

Fig. 9. Thin-layer chromatography of lipids. Silica Gel F_{254}, hexane:diethylether:acetic acid (80:20:1.5, by vol)
A: Control, serum plus reagent without C.E.H. and C.O. *B:* Test, serum plus reagent shown in Table 1. *C:* Standards of free cholesterol and cholesterol palmitate in reagent without C.E.H. and C.O.

lowed to develop. The positions of the lipids were identified by using free cholesterol and cholesterol palmitate in the same reagent but without cholesterol ester hydrolase or cholesterol oxidase. As shown in Figure 9, sterol esters are hydrolyzed and free cholesterol is oxidized by the reagent under the prescribed conditions. Triglycerides in sample were also hydrolyzed, presumably by lipase in the cholesterol ester hydrolase. Initial studies in which cholesterol ester hydrolase was used were done with material isolated in our laboratories from hog pancreatin. When more nearly pure material became available from Ames Laboratories, it was subsequently used.

We have evaluated several indicator reactions for the measurement of the hydrogen peroxide produced, including those described by Gochman and Schmitz (*16*) such as *o*-dianisidine, 4′,4′,4″-methylidynetris(N,N-dimethylaniline), 3-methyl-2-benzothiazolinone hydrazone/*o*-toluidine, 3-methyl-2-benzothiazolinone hydrazone/N,N-dimethylaniline, as well as ABTS [2,2′-azino-di-(3-ethylbenzthiazolin-sulfonate-6) diammonium salt] (*17*). Of the chromogens evaluated, the one described in the present method was considered best in terms of solubility, sensitivity, low blank, and noninhibition of the enzymatic reactions.

Effect of Reducing Compounds

A Metrix normal control serum was reconstituted to half of its specified volume, and aliquots were mixed with equal volumes of solution containing the material to be tested, to give the final serum concentration shown in Table 2. The results show little interference by the compounds studied.

Specificity Studies

The specificity of the present method was assessed by assaying isopropanol solutions of several sterols and comparing the results with those obtained by

Table 2. Effect of Reducing Compounds on Results of Present Method

Reducing compound	Concentration in serum	Cholesterol	Difference, %
	mg/100 ml		
None	...	188	...
Urea	60	189	+0.5
Ascorbic acid	5	184	−2.1
Creatinine	11	189	+0.5
Glucose	500	190	+1.0
Bilirubin	15	186	−1.0
Uric acid	50	191	+1.6
Hemoglobin	200	192	+2.1
Sodium bromide	10	187	−0.5

Table 3. Comparison of Substrate Specificity of Present Method with That of Abell et al. (2)

Sterol[a]	% of sterol measured as cholesterol		Cholesterol error in normal serum, mg/100 ml[b]	
	Present method	Abell	Present method	Abell
5α-Cholestan-3β-ol	82	34	4.1	1.7
7-Dehydrocholesterol	66	160	13.2	32.0
5-Cholesten-3β-20α-diol	82	46	—	—
7-Cholesten-3β-ol	80	154	2.4	4.6
Ergosterol	60	130	—	—
		Total	19.7	38.3

[a] The concentrations used were as follows, per deciliter: 5α-cholestan-3β-ol, 50 mg; 7-dehydrocholesterol, 100 mg; 5-cholesten-3β-20α-diol, 50 mg; 7-cholesten-3β-ol, mg 50; ergosterol, 10 mg.
[b] Calculation of these values based on normal serum content of 5 mg of 5α-cholestan-3β-ol, 20 mg of 7-dehydrocholesterol, and 3 mg of 7-cholesten-3β-ol per deciliter (17).

the method of Abell et al. (2). The results are shown in Table 3. 5α-Cholestan-3β-ol, 7-dehydrocholesterol, and 7-cholesten-3β-ol are found in normal serum at concentrations of 5, 20, and 3 mg/dl, respectively (18).

Precision of the Method

Using native human sera under routine hospital conditions, we found a within-day precision (CV) of 0.50% for a cholesterol concentration with a mean value of 384.4 mg/dl and a standard deviation of 1.91, and a CV of 1.0% for a concentration with a mean value of 46.9 mg/dl and a standard deviation of 0.48. Assays of two specimens of native human sera over a five-day period showed a CV of 0.99% (mean, 386.5 mg/dl; SD, 3.84) and a CV of 3.0% (mean, 48.2 mg/dl; SD, 1.45).

Performance

Native sera samples were assayed by using the AutoAnalyzer-II with isopropanol extraction, the SMA 12/60 without extraction, and by the method of Abell et al. (2). The results obtained, as compared with those by the present method, are shown in Figures 10, 11, and 12, respectively. The calculated linear regressions and correlation coefficients indicate excellent agreement between the present method and the respective methods compared with it. The comparison of results by the present method with those from the SMA 12/60 shows more scatter than the other comparisons, and is probably due to inaccuracies in this direct method.

Discussion

The cholesterol method described here represents the first attempt to incorporate the specificity of a totally enzymatic procedure into a single aqueous reagent. The method has good sensitivity, does not require extraction or sample dilution, and avoids the use of harsh mineral acids. The reagent in solution is stable for 8 h at room temperature (25 °C) and 24 h if kept at 4 °C. The method may be run manually or easily adapted for automated use.

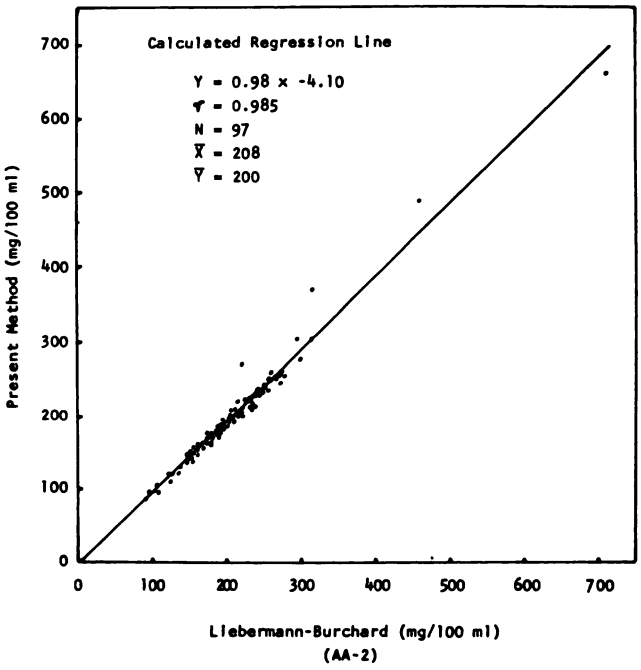

Fig. 10. Correlation of results by automated Liebermann–Burchard (AA-2) and present method

Richmond has demonstrated the specificity of cholesterol oxidase by comparing the rates of oxidation of several cholesterol analogs with that of cholesterol at the same molarity (10). From his data, it was shown that cholesterol oxidase was specific for 3β sterols and required a double bond in the Δ^5 or Δ^4 position of the sterol. He assumed that the Δ^4-3-oxo products formed had the same molar absorbance as Δ^4-cholestenone. Of the cholesterol analogs tested, all showed some oxidation by cholesterol oxidase with the exception of 5α-cholestan-3β-ol and 5β-cholestan-3β-ol. Flegg (9) performed similar experiments, using 5α-cholestan-3β-ol, 7-dehydrocholester-

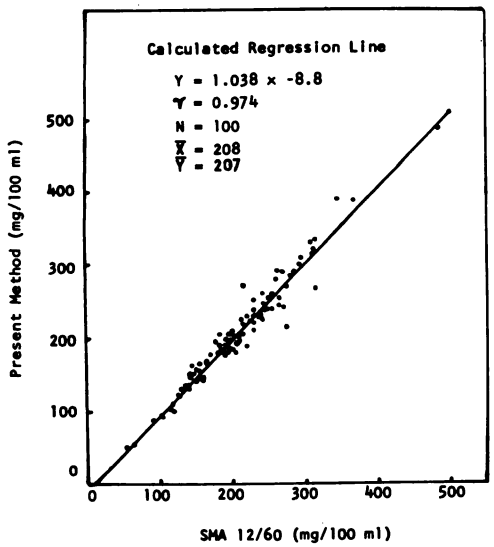

Fig. 11. Correlation of results by SMA 12/60 and present method

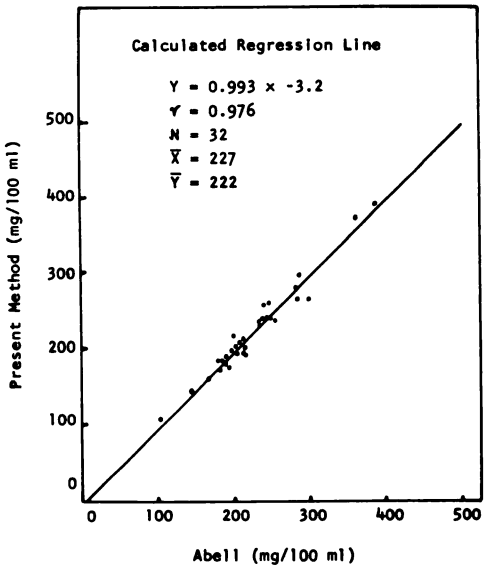

Fig. 12. Correlation of results by the method of Abell and present method

ol, ergosterol, and 20α-hydroxycholesterol, and found that all were oxidized by cholesterol dehydrogenase except 5α-cholestan-3β-ol. However, when Flegg tested the ketones formed by assay with dinitrophenylhydrazine, all showed reactivity including the 5α-cholestan-3β-ol. The present method does not rely on the molar absorptivity of the reaction product or its wavelength maximum, but measures the hydrogen peroxide produced. While the cholesterol analogs measured in this study all showed some positive error, the present method gave less error than did the method of Abell et al. for sterols present in normal serum with the exception of 5α-cholestan-3β-ol. Although absolute specificity for cholesterol is not achieved by the present method, it is more specific than nonenzymatic cholesterol assays. The present method is attractive in terms of better specificity, precision, and simplicity, but it must await the inevitable scrutiny of detailed evaluations such as those described earlier (*1, 4*).

We thank Dr. Nathan Gochman, Clinical Laboratories, VA Hospital, San Diego, Calif. for the assay of split serum samples on the AutoAnalyzer-II, and Dr. Chester Sutula of Ames Laboratories, Elkhart, Ind., for the generous supply of cholesterol ester hydrolase.

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