Enzymatic assay for cholesterol ester hydrolase activity

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Summary A rapid and accurate method is described for the assay of cholesterol ester hydrolase (CEH) activity. Aliquots of the enzyme–substrate incubation mixture are extracted into isopropanol. The free cholesterol concentration in each extract is determined enzymatically using a single aqueous reagent containing cholesterol oxidase and peroxidase. The free cholesterol remaining after the cholesterol ester hydrolase-catalyzed esterification is converted to Δ^4-cholestenone and hydrogen peroxide (H_2O_2); peroxidase couples H_2O_2 with phenol and 4-amino-antipyrine to yield a stable rose-colored product absorbing at 500 nm. The method is highly reproducible and the values correlate well with those obtained with the chromatographic radioassay of CEH activity.

Supplementary key words cholesterol · cholesterol oxidase · peroxidase · 4-amino-antipyrine

During studies on the purification of pancreatic cholesterol ester hydrolase, a rapid and accurate color-

Abbreviations: CEH, cholesterol ester hydrolase; TLC, thin-layer chromatography; LCAT, lecithin cholesterol acyl transferase; BSA, bovine serum albumin.

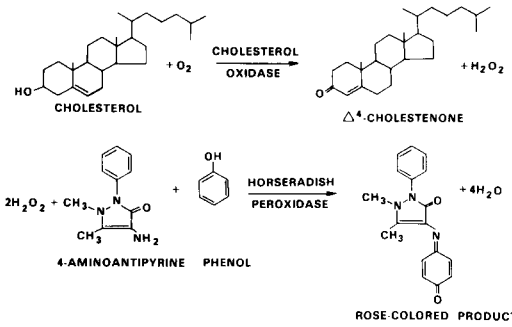

Fig. 1. Reaction scheme for the enzymatic measurement of free cholesterol.

imetric procedure for determining changes in free cholesterol concentration during enzymatic synthesis of cholesterol esters has been developed. The activity of this enzyme has been commonly measured by colorimetric assay (1–3) or radioassay (4, 5) by determination of the rate of appearance (cholesterol ester → cholesterol + fatty acid) or disappearance (cholesterol + fatty acid → cholesterol ester) of free cholesterol during the reversible reaction or by the appearance of fatty acid during the former reaction. These techniques require preparation of a lipid extract, and those that measure free cholesterol require separation of free from esterified cholesterol by digitonin precipitation or by thin-layer chromatography. The precipitation of free cholesterol as the digitonide requires several hours, complete retention of the digitonide through several wash steps is difficult, and the final colored product is unstable. The separation of free and esterified cholesterol by thin-layer chromatography requires less time, but the free and esterified cholesterol must be extracted from the thin-layer adsorbant for either colorimetric determination or prepared for radioactive counting if a radioassay is to be employed. The technique that measures the release of labeled fatty acid (5) is reported to be rapid and reliable; however, caution must be exercised in the preparation of the substrate emulsion to insure saturating levels without altering the activity of those sterol ester hydrolases that are substrate inhibited (5).

The proposed assay utilizes cholesterol oxidase for conversion of total cholesterol to Δ^4-cholestenone and hydrogen peroxide; and peroxidase for the oxidation coupling of peroxide with phenol and 4-aminoantipyrine to yield a colored product. These reactions are shown in **Fig. 1.** Recently, methods for the determination of serum total cholesterol, utilizing cholesterol oxidase and peroxidase, have been reported (6, 7).

Materials and methods

Cholesterol oxidase (E.C. 1.1.3.6) (15 U/mg) and peroxidase (E.C. 1.11.1.7) (250 U/mg) were obtained from Boehringer Mannheim Corporation, Indianapolis, IN. Other chemicals and sources were as follows: cholesterol, Serdary Research Laboratories, London, Ontario; [4-^{14}C]cholesterol, Amersham/Searle Corp., Arlington Heights, IL; phenol, Mallinckrodt Chemical Works, St. Louis, MO; 4-amino-antipyrine, Eastman, Rochester, NY; sodium azide, isopropanol (Spectranalyzed), diethylether (peroxide-free), and Triton X-100, Fisher Scientific Company, Pittsburgh, PA; sodium taurocholate, Grand Island Biological Company, Grand Island, NY; and oleic acid, Sigma Chemical Company, St. Louis, MO. The samples of cholesterol ester hydrolase (CEH) at various steps of purification from rat pancreas were prepared in our laboratory (8).

The complete cholesterol reagent is prepared to contain in 1 l of 0.01 M potassium phosphate buffer, pH 7.0:5.2 mM sodium taurocholate; 2.0 mM sodium azide; 14.0 mM phenol; 0.82 mM 4-amino-antipyrine; 0.5 ml of Triton X-100; 90 units of cholesterol oxidase; and 33,000 units of peroxidase. This reagent remains stable for at least one month when stored at 4°C.

The substrate mixture used for assay of cholesterol ester hydrolase has been described previously (8) and contains 3 mg of cholesterol, 13.1 mg of oleic acid, 17.7 mg of sodium taurocholate, and 6 mg of bovine serum albumin (fraction V) (BSA) per ml of 0.154 M potassium phosphate buffer, pH 6.2. The cholesterol and oleic acid are added to a Potter-Elvehjem type homogenizing tube in ether solution and the ether is evaporated under a stream of nitrogen. The potassium phosphate buffer, containing BSA and sodium taurocholate, is added, and the contents of the tube are homogenized with a motor-driven Teflon pestle.

We have previously evaluated the assay procedure for the esterification of cholesterol with oleic acid (9). Under the conditions reported here, the substrate concentrations are saturating, the reaction rate is linear with incubation time within the limits of 5 and 50% esterification, and in this range, there is a direct proportionality between enzyme concentration and reaction rate. Therefore, in the present assay, the activity of cholesterol ester hydrolase solutions is adjusted, if necessary, by dilution with 0.154 M potassium phosphate buffer, pH 6.2, to fall within the range of esterification mentioned above. The solutions of cholesterol ester hydrolase are added to 3 ml of substrate mixture to give a total reaction volume of 6 ml. Incubations are carried out at 37°C in a Dubnoff metabolic shaker. During the enzymatic reaction, three 1-ml aliquots of the incubation medium are withdrawn between 2 and 20 min of incubation time and added to a 5-ml volumetric flask which contains approxi-

mately 3 ml of isopropranol. The volumes of substrate and enzyme in the sterol ester hydrolase assay, the size of the aliquots withdrawn, and the volume of the isopropanol extract can be proportionately decreased to accommodate specific needs. In our system, the availability and activity of the enzyme were not limiting; therefore, we chose to employ the larger volumes which were more convenient to handle.

After addition of the aliquots to the isopropanol, the mixture is brought to a rolling boil, allowed to cool, and diluted with isopropanol to a total volume of 5 ml. An 80-μl aliquot of the cholesterol extract is added to 3 ml of the complete cholesterol color reagent. Development of the rose color is complete in 10 min at room temperature and is stable for at least 90 min at room temperature. The absorbance is measured against a reagent blank at 500 nm. One unit of CEH activity is defined as the amount of enzyme catalyzing the disappearance of 1 μmol of cholesterol per hr.

Results

Standard curve. Cholesterol standards were prepared in isopropanol to contain 12.5, 25, 50, 75, and 100 mg of cholesterol per dl. Eighty μl of each cholesterol standard was added to 3.0 ml of the complete color reagent. The linear relationship between cholesterol concentration and absorbance is shown in **Fig. 2.**

Accuracy. A comparison of the determination of CEH activity (0–200 units) with the chromatographic radioassay of Vahouny et al. (4) and the enzymatic assay described in the present paper is given in **Fig. 3.** The calculated linear regression and correlation coefficient (0.986) indicate excellent agreement between the present method and the chromatographic radioassay.

Reproducibility. A cholesterol ester hydrolase assay was carried out as described above. Aliquots of the

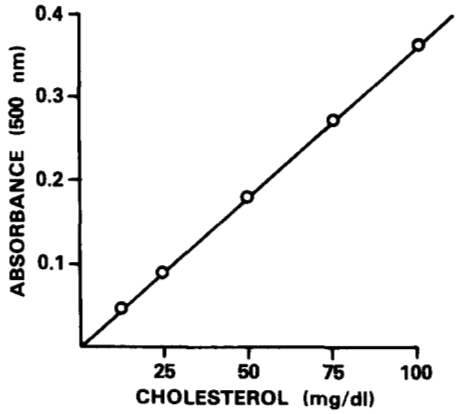

Fig. 2. Standard curve for determination of free cholesterol. Each point is the mean of three determinations.

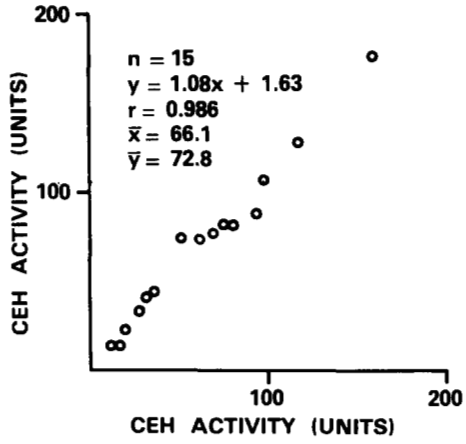

Fig. 3. Comparison of the proposed method with the micro TLC radioassay (4) for the determination of CEH activity. y-axis, present method; x-axis, radioassay.

enzyme–substrate mixture were removed at 6, 15, and 19 min and each was extracted in isopropanol. The level of cholesterol in each of three isopropanol extracts was determined in triplicate with nine different preparations of the complete cholesterol reagent. The calculated mean percentage esterification of cholesterol was 11.6 ± 2.2 (SD), range 9.1–16.2; 28.5 ± 1.1 (SD), range 27.3–30.3; 35.0 ± 2.4 (SD), range 30.1–39.4.

Precision. The precision of the method was determined by repeated measurement (10 determinations) of the level of cholesterol in four cholesterol standard solutions prepared in isopropanol. At cholesterol concentrations of 15, 30, 60, and 90 mg/dl (12, 24, 48, and 72 μg cholesterol/80 μl), the following mean values and standard deviations were determined, respectively: 14.9 mg/dl ± 1.10; 28.7 mg/dl ± 1.07, 60.3 mg/dl ± 0.91; and 90.1 mg/dl ± 1.87. The first two concentrations, 12 μg and 24 μg per 80 μl, correspond to the levels of cholesterol measured when 50% and no esterification have occurred in the assay, respectively.

Discussion

The present report describes an enzymatic assay for the measurement of cholesterol ester hydrolase activity. This method depends upon the enzymatic determination of free cholesterol concentration at timed intervals during the CEH-catalyzed esterification. Similar enzymatic methods have been recently described for the determination of serum cholesterol (6, 7) and for the esterification of free cholesterol in human plasma by the action of LCAT (10). The major advantage of the method reported here is the rapidity of the assay, i.e., approximately 30 min are required from the time of sampling the enzyme–substrate

mixture through absorbance measurement. The time required for enzyme assay becomes increasingly crucial during the course of the purification of the extremely labile CEH. The method is suited to handling multiple determinations with no necessity for careful timing since the final color developed is stable for at least 90 min. The precision and reproducibility of the method are comparable to that for the chromatographic radioassay (4), and the precision better than that for the chemical measurement of free cholesterol (1).

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REFERENCES

1. Sperry, W. M., and M. Webb. 1950. A revision of the Schoenheimer-Sperry Method for cholesterol determination. *J. Biol. Chem.* **187:** 97–106.
2. Brown, H. H., A. Zlatkis, B. Zak, and A. J. Boyle. 1954. Rapid procedure for determination of free serum cholesterol. *Anal. Chem.* **26:** 397–399.
3. Vahouny, G. V., C. R. Borja, R. M. Mayer, and C. R. Treadwell. 1960. A rapid, quantitative determination of total and free cholesterol with anthrone reagent. *Anal. Biochem.* **1:** 371–381.
4. Vahouny, G. V., C. R. Borja, and S. Weersing. 1963. Radioactive and analytical determination of free and esterified cholesterol following micro thin-layer silicic acid chromatography. *Anal. Biochem.* **6:** 555–559.
5. Pittman, Ray C., John C. Khoo, and David Steinberg. 1975. Cholesterol esterase in rat adipose tissue and its activation by cyclic adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **250:** 4505–4511.
6. Allain, C. C., L. S. Poon, S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:** 470–475.
7. Tarbutton, P. N., and C. R. Gunter. 1974. Enzymatic determination of total cholesterol in serum. *Clin. Chem.* **20:** 724–725.
8. Calame, K. B., L. Gallo, E. Cheriathundam, G. V. Vahouny, and C. R. Treadwell. 1975. Purification and properties of subunits of sterol ester hydrolase from rat pancreas. *Arch. Biochem. Biophys.* **168:** 57–65.
9. Hyun, J., H. Kothari, J. Mortensen, C. R. Treadwell, and G. V. Vahouny. 1969. Purification and properties of pancreatic juice cholesterol esterase. *J. Biol. Chem.* **244:** 1937–1945.
10. Patsch, W., S. Sailer, and H. Braunsteiner. 1976. An enzymatic method for the determination of the initial rate of cholesterol esterification in human plasma. *J. Lipid Res.* **17:** 182–185.