Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells

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Summary Procedures for the determination of free and total cholesterol in lipid extracts or sonicates of 10^4 cultured human skin fibroblasts are described. The method for free cholesterol employs cholesterol oxidase to generate H_2O_2 and peroxidase to catalyze the reaction of H_2O_2 with *p*-hydroxyphenylacetic acid to yield a stable fluorescent product. Cholesterol ester hydrolase is included for determination of total cholesterol. When samples of sonified cell suspensions are used directly, the extraction of lipids is avoided, permitting one person to carry out analyses of 30 or more subcultures in one day.

Supplementary key words Cholesterol assay · cholesterol oxidase · cholesterol ester hydrolase · fibroblasts · tissue culture

For studies of cholesterol transport and metabolism, we needed to quantify free and esterified cholesterol in single dishes of cells grown in culture. It was important to have a sensitive assay with which to analyze large numbers of samples relatively rapidly. For many reasons, some of which have been recently discussed by Ferrel, Repola, and Batsakis (1), we preferred not to use gas-liquid chromatography. Allain et al. (2) have described a procedure in which H_2O_2 generated in the specific enzymatic oxidation of cholesterol is used in a reaction catalyzed by peroxidase to yield a chromogen. We report here a modification of this method in which *p*-hydroxyphenylacetic acid is used in the peroxidase-catalyzed reaction to yield a stable product of high relative fluorescence described by Guilbault, Brignac, and Juneau (3). For determination of total cholesterol, cholesterol ester hydrolase is included in the assay system. Using the methods described here, the free and total cholesterol content of 10^4 fibroblasts can be rapidly and reliably determined.

Materials

Cholesterol oxidase, horseradish peroxidase, and cholesterol ester hydrolase were purchased from Boehringer-Mannheim Corp., New York; *p*-hydroxyphenylacetic acid and sodium cholate were from Sigma Chemical Co., St. Louis, MO; chloroform ("Spectranalyzed") was from Fisher Scientific Co., Fair Lawn, NY; and cholesterol, cholesteryl oleate, and campesterol were from Applied Science Laboratories Inc., State College, PA.

Procedure using chloroform–methanol extraction

Human skin fibroblasts were grown on 100 × 20-mm plastic petri dishes, washed, and harvested into 13-ml centrifuge tubes with ground glass stoppers as described elsewhere (4). Lipids were extracted from the cells with chloroform–methanol 2:1 (v/v). The extracted lipids, after evaporation of solvent, were dissolved in chloroform and samples for assay were transferred to disposable glass tubes (12 × 75 mm). The extracted cell pellets were used for protein determination (5). Standard solutions of cholesterol and cholesteryl oleate were prepared in chloroform and appropriate samples were distributed to assay tubes. Chloroform was evaporated under N_2, and the tubes were then heated for 20 min in an oven at 105°C. Heating was necessary to remove traces of solvent that would otherwise interfere in the assay.

The composition of the solution used for determination of total cholesterol is shown in **Table 1.** For determination of free cholesterol, the same solution minus cholesterol ester hydrolase was used. Ethanol (95%) was added to dissolve samples in the assay tube. The assay solution was then added, and after incubation for 30 min at 37°C fluorescence was measured in an Aminco-Bowman Spectrophotometer (excitation, 325 nm; emission, 415 nm). Samples containing 50–500 ng of cholesterol were dissolved in 15 μl of 95% ethanol, 135 μl of assay solution was used, and fluorescence was determined in a 3-mm cuvette. Samples containing 0.5–10 μg of cholesterol were dissolved in 0.2 ml of ethanol, 2 ml of assay solution was added, and fluorescence was measured in a 1-cm cuvette. The enzymatic reactions were completed in 15 min under assay conditions. After incubation, there was some increase in fluorescence but at a rate such that there was essentially no detectable change during the first hour.

As shown in **Fig. 1** for the assay carried out in a total volume of 150 μl, fluorescence was proportional to amount of cholesterol between 50 and 500 ng whether free or esterified cholesterol was assayed. Similarly, fluorescence was proportional to cholesterol between 0.5 and 10 μg in the assay with a total volume of 2.2 ml (data not shown). In the assay (2.2 ml) for free cholesterol (i.e., in the absence of cho-

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TABLE 1. Composition of solution for assay of total cholesterol in lipid extracts

Stock Solution	Relative Proportion
Potassium phosphate buffer 0.1 M, pH 7.4	8
Cholesterol oxidase, 1 U/ml, in buffer[a]	2
Peroxidase (horseradish), 10 U/ml, in buffer[a]	2
Cholesterol ester hydrolase, 0.1 U/ml, in buffer[a]	2
Triton X-100, 0.5%	1
Sodium cholate, 20 mM	1
p-Hydroxyphenylacetic acid, 4 mg/ml	3

[a] Buffer is potassium phosphate, 0.1 M, pH 7.4.

Stock solutions were stored at 4°C and were stable for at least one month. The assay solutions were prepared on the day of use. For determination of free cholesterol, cholesterol ester hydrolase was omitted from the assay mixture and a total of 10 volumes of phosphate buffer was added.

lesterol ester hydrolase), as much as 8 μg of cholesteryl oleate yielded no detectable fluorescent product.

The amounts of total and free cholesterol as determined in extracts of cultured human skin fibroblasts were directly proportional to the volume of extract assayed. This is shown in **Fig. 2** for the assay carried out in a total volume of 2.2 ml.

The enzymatic assay was directly compared by analyses of replicate samples from the same tissue extract using gas-liquid chromatography. Values obtained with the two methods differed by less than 4% as shown in the experiments presented in **Table 2.**

Procedure using cell homogenate

Fibroblasts were washed and transferred to 12 × 75-mm glass tubes. After centrifugation and removal of the medium used for harvesting cells, 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, was added to the cell pellet. Each sample was sonified for 1 min using the microtip of the sonifier (Branson Instruments Inc., Stamford, CT). Samples (usually 0.1 ml) of the sonicates were transferred to 12 × 75-mm tubes for determination of free and total cholesterol. (Other samples were used for assay (4) of protein.) To each tube was added 20 μl of a solution of 20 mM sodium cholate and 1% Triton in the assay buffer followed by 25 μl of 95% ethanol. The appropriate enzymes and *p*-hydroxyphenylacetic acid were then added in a volume of 0.2–0.5 ml, depending on the amounts of cholesterol in samples, to achieve the same final concentrations of components used in the procedure

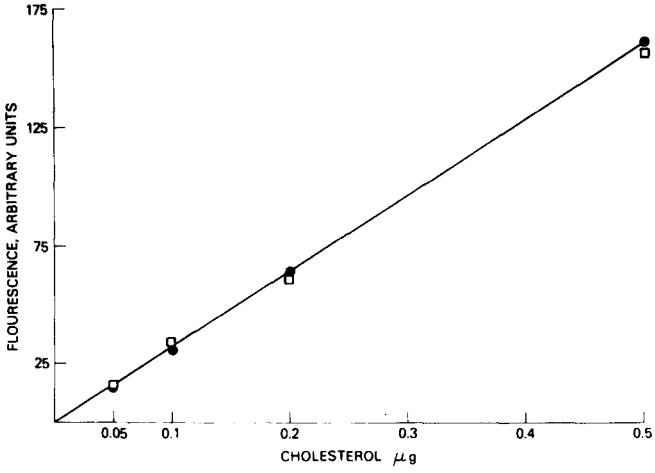

Fig. 1. Fluorescence vs. amount of free or esterified cholesterol in assay (total volume 150 μl). (●) Free cholesterol; (□) esterified cholesterol.

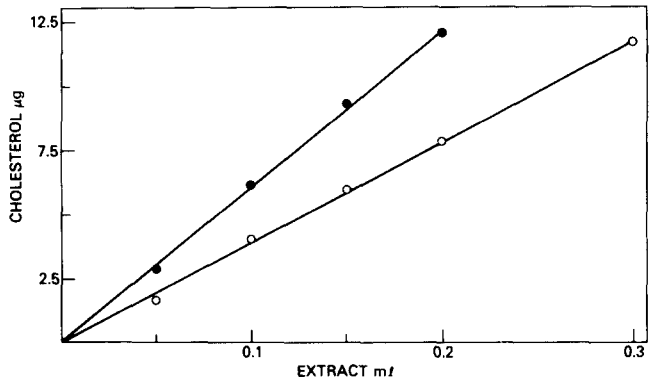

Fig. 2. Free and total cholesterol in a lipid extract of human skin fibroblasts. (○) Free cholesterol; (●) total cholesterol. Cells were grown to confluency, then incubated for 3 days in Eagle's minimal essential medium (6) containing 5% (v/v) of hypercholesterolemic rabbit serum. Assay volume was 2.2 ml.

TABLE 2. Comparison of enzymatic and gas-liquid chromatographic (GLC) methods: free and total cholesterol in extracts of cultured fibroblasts

Exp. No.	Cholesterol Content[a] Enzymatic (A)	GLC (B)	Difference (A − B) ÷ B × 100
1 Free	91.6 ± 0.7	88.6 ± 0.7	+3.4%
Total	95.0 ± 0.6	97.6 ± 0.1	−3.0%
2 Free	51.5 ± 2.3	51.6 ± 0.4	−0.2%
Total	55.6 ± 1.1	54.0 ± 0.1	+3.0%

[a] Mean ± SEM of three determinations.

Replicate samples of lipid extracts of cultured fibroblasts were analyzed by the enzymatic method described here (total volume, 2.2 ml) or by GLC. Campesterol was used as an internal standard in the latter procedure as suggested by Stein, Vanderhoek, and Stein (7). Samples for determination of total cholesterol by GLC were saponified (8). For chromatography we used 1% OV-17 in a 4-ft column (Supelco). In Exp. 1 the extract was prepared from fibroblasts grown for 6 days in Eagle's minimal essential medium (6) containing 10% fetal calf serum and in Exp. 2 from fibroblasts grown for 24 hr in that medium and then for 6 days in the same medium without serum.

for analysis of lipid extracts. Assays were incubated for 1 hr at 37°C, and fluorescence was measured as described above.

We compared the direct assay of sonicates with the method employing chloroform–methanol extraction using cells that had been labeled by incubation for 48 hr with [^{14}C]cholesterol. Based on radioassay and total cholesterol determinations of replicate samples of sonicates and of chloroform extracts, it was established that the values obtained by the two procedures agreed within less than 5%. Considering the potential for error in the procedure utilizing solvent extraction, which depends on the quantitative recovery of both extracted lipids and precipitated protein, we believe that the modified assay using sonified cells is preferable for most purposes. With it one person can readily complete analyses of free and total cholesterol in 30 or more subcultures in one day.

Addendum

After this report was submitted, the editors made available to us a copy of the publication by Heider and Boyett (9) which describes a procedure similar to ours in many ways. The substantive differences appear to be *1*) the method of preparation of cell extracts for analysis and *2*) the pH of the solution in which fluorescence is determined. We see no particular advantage to the isopropanol extraction of cells suggested by Heider and Boyett (9) and, in fact, for most studies we now carry out assays using sonified cells. We had explored the possibility of increasing the sensitivity of the assay by increasing the pH of the system before measuring fluorescence. We did not find a range in which fluorescence was relatively independent of pH and concluded that the possibility of variations in pH resulting from the additional alkalinization step made it not particularly advantageous, since the sensitivity of the assay as described, which is similar to that of the procedure of Heider and Boyett (9), is quite adequate for our purposes. Clearly, for some applications, it may be useful to increase the pH of the system before measuring fluorescence.

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