A micro dibromide procedure for purifying radioactive cholesterol*

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» The interpretation of isotope experiments on cholesterol metabolism will be subject to uncertainty until the contribution of related companion sterols can be evaluated. The error in assuming homogeneity of the initially isolated sterol can be considerable (1 to 4). For the further purification of the isolated sterol, a micro dibromide procedure was needed. For the manipulation of 1-mg amounts of cholesterol the usual macromethods (2, 5) cannot be used. The scaled-down quantities of glacial acetic acid and cold methanol of the macromethod still dissolve small amounts of dibromide. Consequently, the macromethod was modified, and the resulting micromethod previously reported (6) is presented in detail.

MATERIALS AND METHODS

The chemicals used in these experiments were C.P. or reagent grade and required no further purification. However, the condition of the zinc granules was found to be critical. Upon standing, the metal becomes coated with carbonate, which seriously impairs its reactivity to acetic acid. The zinc, therefore, was washed with concentrated HCl followed by distilled water until evolution of hydrogen stopped. The metal was then used immediately.

Cleavage of the Cholesterol-Glycoside Complex. Cholesterol isolated from biological material was recovered by precipitation with digitonin (3, 4) or with the new glycoside tomatine (7). Before bromination of cholesterol it was necessary to cleave this insoluble complex. The digitonide was readily cleaved by the pyridine method of Schoenheimer and Dam (8). The tomatinide can be split by dissolving it in dimethylformamide as reported by Schulz and Sander (9). In our hands the latter method was not reliable and the following method of hydrolysis was introduced.

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The cholesterol tomatine complex representing 1 mg of cholesterol was dissolved in 1.0 ml of acetic acid and treated with an equal volume of 40% NaOH (w/v). The solution was thoroughly mixed and heated on a water bath (55° to 60°) for 30 minutes. The solution was then extracted three times with 3 to 4 ml portions of diethyl ether. The ether fractions were combined and carefully evaporated (35° to 40°) to 3 to 4 ml, and the excess acetic acid in the ether was neutralized with 10% sodium hydroxide solution using phenolphthalein as an indicator. The ether layer containing the free sterol was separated from the slightly basic aqueous phase and concentrated to 1 ml.

Bromination of Micro Quantities of Cholesterol. To the above ether solution, liquid bromine was added until it assumed a slight orange color. An excess of bromine interferes with the formation and isolation of the dibromide derivative. After 1 hour at room temperature, 3 ml of acetic acid was added, and the tubes were immersed in an ice bath. When the solution was thoroughly chilled, ice water was added until the first appearance of a precipitate. Crystallization was then allowed to continue at room temperature for 1 or 2 hours, or preferably overnight.

Debromination of 5α,6β-Dibromo Cholestane-3β-ol. The solid dibromide derivative was separated from the solution by centrifugation, washed twice with 3 ml of distilled water, and dissolved with 3.0 ml glacial acetic acid. After addition of 10 granules of zinc, the reaction mixture was heated on a water bath (60°) for 30 to 60 minutes and then cooled to room temperature. The solution was separated from the unreacted zinc by decantation and the granules washed twice with 1-ml portions of acetic acid. The filtrate and combined washings were neutralized to phenolphthalein with 10% sodium hydroxide, and then extracted with several portions of ether. The ether solution was evaporated to near dryness, and the purified cholesterol dissolved in 2 to 3 ml of acetone-alcohol-ether 4/4/1 (v/v). In order to avoid quenching by residual halogen in our liquid scintillation counting system, the purified cholesterol was reisolated and assayed as the tomatinide as previously described (7). Starting with a 1.0 mg sample, 30% to 4% yields were reproducibly recovered. With quantities greater than 1.0 mg, the percentage yields were correspondingly higher.

DISCUSSION

In order to brominate cholesterol isolated either as the digitonide or tomatinide, it was necessary to split the complex by using alkaline hydrolysis. The concentration of alkali and the conditions under which cleavage was brought about were sufficiently mild so as not to cause noticeable degradation, as demonstrated by recovery of the sample in high yields. The amount of bromine used for the halogenation was critical. If the solution became too dark an orange, it was not possible to isolate the dibromide derivative. For precipitation of the dibromide derivative, it was imperative that cold water be added dropwise; too little as well as too much water resulted in poor yields. Under these conditions of water addition, nonbrominated cholesterol was soluble and could not be precipitated. To eliminate the possibility that nonbrominated cholesterol had co-precipitated with the dibromide derivative, the precipitated derivative was isolated, redissolved in acetic acid, and the presence of free sterol tested with the Liebermann-Burchard reagent. Failure to develop color under these conditions verified that the precipitated compound contained less than 30 mg cholesterol.

The purification of radioactive cholesterol via the dibromide is considered the best procedure for eliminating higher counting companions of the sterol (1). In agreement with Schwenk and Werthessen, a single bromination and reconversion to cholesterol was usually sufficient to establish radiochemical purity of the isolated sterol.

Table 1 represents data typical of studies on humans.

TABLE 1. RADIOCHEMICAL PURITY OF CHOLESTEROL ISOLATED FROM HUMAN PLASMA AS THE DIGITONIDE

Time After I.V. Acetate 1-C^{14}*	Initial Specific Activity	Macro† Dibromide	Micro‡ Dibromide
Minutes	*mc/g*	*mc/g*	*mc/g*
10	0.057	0.026	0.030
20	0.120	0.103	0.105
30	0.241	0.235	0.227
60	0.345	0.340	0.343

* 100 μc.
† >100 mg (see Ref. 1).
‡ <10 mg.

The macro dibromide method (1) and our micromethod are in good agreement. The use of the micromethod for the purification of H^3-labeled cholesterol isolated as the tomatinide is reported elsewhere (7)

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