A SIMPLIFIED BIOLOGICAL SYNTHESIS OF LABELED CHOLESTERYL ESTERS.

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SUMMARY

A simplified procedure is described for the synthesis and purification of labelled cholesteryl esters. The esters are synthesized enzymatically from (4-^{14}C) cholesterol and free fatty acids.

The separation of the lipids from aqueous medium is simplified by lyophilizing the reaction mixture at equilibrium. The lipid components of the freeze-dried powder are separated by silicic acid chromatography. The radiochemical purity of the product was shown to be greater than 98%.

INTRODUCTION

Chemical synthesis of cholesteryl esters can be achieved by heating the sterol and the appropriate fatty acid in an atmosphere of CO_2 (1), or in the presence of benzene sulfonic acid (2). Heating cholesterol and a fatty acyl chloride in the presence of

pyridine also gives a good yield of ester (3,4). For the preparation of unsaturated fatty acid esters a sodium ethoxide catalyzed ester interchange reaction involving cholesteryl acetate and the desired fatty acid methyl ester is most useful (5). None of the existing chemical methods is convenient for microscale synthesis of cholesteryl esters of high specific activity. Using enzymatic synthesis (pancreatic cholesteryl ester hydrolase), (6,7), it is possible to obtain cholesteryl esters in quantities as low as 1 mg.

One of the important requirements for the effective production of radioactive cholesteryl ester by enzymic techniques is the extraction and purification of the ester. When the enzymic reaction reaches equilibrium, the cholesterol, free fatty acid and synthesized cholesteryl ester are extracted with organic solvents followed by solvent evaporation and separation of the components by silicic acid column chromatography. This widely-used procedure is not only time-consuming but our experience has been that the fatty acid becomes oxidized during extraction producing an insoluble complex of lipids. Consequently the recovery of the ester is poor. We believe that the technique described in this communication, namely the lyophilization of the reaction mixture at equilibrium, circumvents these difficulties and is a rapid and efficient procedure for the separation of the lipid components. The freeze-dried powder is extracted with a small quantity of petroleum ether (boiling range 60-110°) and free and esterified [4-^{14}C]cholesterol are separated by silicic acid column chromatography. In our studies most of the

radioactivity is recovered after lyophilization. Using the described method, 99% pure, high specific-activity [4-^{14}C]cholesteryl oleate, [4-^{14}C]cholesteryl linoleate, [4-^{14}C]cholesteryl linolenate and [4-^{14}C]cholesteryl arachidonate have been prepared in radiochemical yields of 61%, 57%, 59% and 56%, respectively.

EXPERIMENTAL

Chemicals: All chemicals were purchased from Sigma Chemical Company, St. Louis, Mo., or General Biochemical Co., Chagrin Falls, Ohio. Cholesterol and free fatty acids were checked for purity by thin-layer chromatography. [4-^{14}C]cholesterol (specific activity, 58.5 mCi/n mole) was purchased from New England Nuclear Corp., Boston, Mass.

All activity measurements were made using a Packard Tricarb Scintillation Spectrometer, Model 3375, with an efficiency of 75% and background counts of 30-35 CPM. The samples were counted in a liquid scintillation mixture containing 2,5-diphenyloxazole, (PPO), 4 g, 1,4-phenylenebis(4-methyl-5-phenyloxazole), (DMPOPOP), 0.1 g made up to 1 liter with distilled toluene.

Enzyme: Cholesterol ester hydrolase, (EC3.1.1.13). An enzyme solution was prepared by extracting acetone-dried powder of beef pancreas (obtained from Sigma Chemical Co.). The enzyme was brought into solution by suspending 1 g of the powder in 10 ml of 0.1 M NH_4Cl solution at 4°. The suspension was stirred for 30 min and then centrifuged at 10,000 X g for 10 min. The clear supernatant contained 10 mg protein per ml.

Reaction Mixture: The reaction mixture was prepared by emulsifying 3 mg (0.0077 m mole) [4-^{14}C]cholesterol (0.5 μCi), 6 mg (0.021 m mole) oleic acid and 8 mg (0.015 m mole) sodium taurocholate in a volume of 0.5 ml 0.15 M phosphate buffer, pH 6.2. The lipid components dissolved in ether were placed in a Potter-Elvehjem homogenizer and after addition of the inorganic components the mixture was homogenized. The ether was removed in a stream of N_2 and the mixture was rehomogenized prior to use. After addition of the enzyme extract (0.5 ml) the reaction was allowed to proceed for 4 hr at 37°.

At the end of reaction period, the mixture was chilled and lyophilized. The freeze-dried powder was extracted with three 5 ml portions of boiling petroleum ether (boiling range 60-110°) and the combined extracts dried under reduced pressure. The residue was dissolved in 1.5 ml of petroleum ether.

Four grams of silicic acid (8) were suspended in 20 ml of methanol and poured in a column 1.5 cm in diameter and 22 cm long. The column was washed in succession with 15 ml portions of acetone, dry ethyl ether and petroleum ether. The sample, dissolved in petroleum ether was placed on the column. The ester was eluted with 1% ethyl ether in petroleum ether. Approximately 80 ml was required to elute [4-^{14}C]cholesterol ester. Upon increasing the ethyl ether concentration first to 5% and then to 10%, unchanged fatty acid and [4-^{14}C]cholesterol were eluted in succession.

RESULTS

The rate of cholesteryl ester synthesis has been shown to be maximal with oleic acid followed by linoleic acid, linolenic acid and arachidonic acid (9). In all cases, however, the equilibrium was reached at about 4 hr of incubation. At equilibrium 60-65% of cholesterol was converted into cholesteryl ester. Five microliters of each reaction mixture was subjected to silicic acid thin-layer chromatography and the radioactivity of separated free and esterified [4-^{14}C]cholesterol was determined. The sum of these two measurements gave the total radioactivity. Similarly, radioactivity was determined for free and esterified cholesterol before and after lyophilization of the reaction mixture at equilibrium. More than 95% of the radioactivity was recovered for the ester from the freeze-dried powder. The recovery of residual free cholesterol was 80-85%.

The lipid mixture extracted from lyophilized powder was separated into its components by silicic acid column chromatography. The ester was eluted with 1% ethyl ether in petroleum ether. Approximately 98% of CPM of ester was recovered in this fraction. The solvent was evaporated under reduced pressure and the residue was dissolved in 1 ml of benzene and checked for purity by silicic acid thin-layer chromatography. The amount of ester cholesterol was determined colorimetrically using the $FeCl_3$ method (10).

This method provides a facile means of synthesizing labelled steryl esters from small quantities of hard-to-obtain sterols or fatty acids. The findings are summarized in Table I.

TABLE I

OVERALL YIELD OF LABELLED CHOLESTERYL ESTERS

Ester	% DPM in Ester Synthesized*	Recovery of Ester After Lyophilization %	Recovery of Ester After Silicic Acid Chromatography %	% DPM in Purified Ester*	Sp. Act. of Ester 10^6 DPM/m mole
Oleate	64.5	96	98	60.6	2608
Linoleate	61.0	95	98	57.0	2526
Linolenate	65.0	94	96	58.5	2636
Arachidonate	59.5	97	97	56.0	2682

*Percent yield based upon equilibrium mixture.

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