J. Lipid Research, January, 1962
Volume 3, Number 1

Preparation of cholesterol esters of long-chain fatty acids and characterization of cholesteryl arachidonate*

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[Received for publication June 30, 1961]

SUMMARY

Cholesterol esters of long-chain saturated fatty acids (C_{12} to C_{22}), as well as those of the C_{18} unsaturated fatty acids (oleic, linoleic, and linolenic), were prepared by the ester interchange method, starting with cholesteryl acetate and the methyl esters of the fatty acids and using sodium ethylate as a catalyst. Cholesteryl arachidonate was synthesized and obtained in crystalline form, and its melting point, optical rotation, and infrared spectrum were recorded. The infrared spectrum of cholesteryl arachidonate showed minor variations from the unsaturated esters of the C_{18} acids. However, the infrared spectra of cholesteryl arachidonate prepared from arachidonate of different sources, one synthetic and the other of natural origin, were identical in all respects. The application of this procedure to the synthesis of labeled cholesterol esters on a microscale was demonstrated by the preparation of cholesteryl oleate-C^{14} from randomly labeled C^{14}-methyl oleate. The purity of the cholesterol esters was established by thin-layer chromatography and other techniques.

A study of the physiological role of cholesterol esters of long-chain fatty acids necessitated the preparation of these compounds in a state of high purity. The existing methods are too complex, time-consuming, and in other ways unsatisfactory, especially for the preparation of cholesterol esters of polyunsaturated and labeled fatty acids. Procedures commonly used for their preparation are esterification of cholesterol with free fatty acids, acid anhydrides, or acid chlorides. Page and Rudy (1) prepared some cholesterol esters of saturated fatty acids by heating cholesterol and the appropriate fatty acid at 200° for 3 to 4 hours in an atmosphere of carbon dioxide. Cataline *et al.* (2) obtained the same esters in 40% to 46% yield by refluxing a mixture of cholesterol and fatty acids in benzene solution for 3 hours at 130° to 140° in the presence of an acid catalyst. These methods have been limited to the preparation of sterol esters of saturated fatty acids and are unsuitable for the unsaturated compounds. Fusion with acids requires relatively high temperature, causing the production of undesirable side products. The reaction using acid catalysts gives poor yields and the purification of the products presents considerable difficulties. The methods involving the use of acid anhydrides or acid chlorides (1, 3, 4, 5) have limitations because of the difficulties encountered in their preparation. Although the anhydrides are mild esterifying agents, their reactivity decreases progressively with increasing molecular weight, the anhydrides of palmitic and higher acids being almost inert (6). Besides, the preparation of anhydrides requires the corresponding acid chlorides (7, 8) or free acids (9). The acid chlorides of saturated fatty acids are obtained in fair yields by the use of conventional chlorinating agents, but the preparation of unsaturated acid chlorides requires expensive reagents and special care and the yields often are not good (10).

Also, the purification of the cholesterol esters by crystallization from organic solvents to remove starting materials or by-products of the reaction, presents considerable difficulties. As a result, there are great differences in the physical properties of cholesterol esters reported by various workers. There are considerable discrepancies in the literature on the reported melting points of the cholesterol esters. The melting point of cholesteryl laurate has been variously reported as 78° (11), 91° (1), 100° (12), and 110° (13).

* Supported by grants from the American Heart Association, National Heart Institute (Grant H-2772[C2]), and the Hormel Foundation. A preliminary communication has appeared elsewhere (*J. Am. Oil Chemists' Soc.* **37**: 685, 1960).

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Extensive turbidity phases have been reported for many other saturated esters (3). In the case of the unsaturated esters, the situation is equally confusing. Page and Rudy (1) listed for cholesteryl linolenate a melting point higher than that for oleate or linoleate. These differences, in some cases, have been explained on the basis of the existence of isomorphic forms (11) and mesomorphic melting transformations (14).

Kuksis and Beveridge (6) refer to the inadequacy of information in the literature on the mesomorphic melting transformations of cholesterol esters, especially in the light of a recent publication on the subject by Gray (14), who could not confirm the mesomorphic melting transformations reported by earlier workers. These discrepancies could be explained on the basis of impure starting materials or unsatisfactory methods for purifying the final products and testing their purity. Recent developments in chromatographic methods have helped greatly to ensure the purity of the starting materials. The same techniques can be used to ensure the purity of the reaction products.

This paper describes in detail a convenient procedure applicable for the synthesis of pure cholesterol esters of both saturated and polyunsaturated fatty acids in good yields by the ester interchange method. Cholesteryl acetate is caused to react with methyl or ethyl esters of long-chain saturated and unsaturated fatty acids in the presence of sodium ethylate catalyst, resulting in the interchange of fatty acid groups. Using this method, the hitherto undescribed cholesteryl arachidonate has also been prepared in good yield and its properties are described. The suitability of this technique for the preparation of isotopically labeled esters in small quantities is illustrated by the preparation of cholesteryl oleate-C^{14}.

Application of a relatively new technique, thin-layer chromatography (TLC), in the course of the synthesis of the cholesterol esters, both as a means of testing their purity and also as a possible method of separating them, is also described.

EXPERIMENTAL METHODS AND RESULTS

Materials. Cholesterol (U.S.P.) (Nutritional Biochemical Corporation) was purified via the dibromide by the method of Fieser (15). The purified material gave a negative color test with the selenium dioxide reagent of Fieser (16). Cholesteryl acetate was prepared by refluxing purified cholesterol with acetic anhydride for 2 hours (17). It melted at 113° to 114°.

The methyl esters of lauric, myristic, palmitic, stearic, arachidic, behenic, oleic, linoleic, and linolenic acids were bought from the Hormel Foundation, Austin, Minnesota, and were found to be highly pure as tested by gas-liquid chromatography as well as conventional methods. A sample of synthetic ethyl arachidonate was obtained from Hoffmann-LaRoche. For purposes of comparison, a sample of 90% pure methyl arachidonate of natural origin (hog liver) was also obtained from the Hormel Foundation. Randomly labeled C^{14}-methyl oleate was a sample that had been isolated from *Chlorella pyrenoidosa* by Mangold and Schlenk (18).

Sodium ethylate was prepared by dissolving small quantities of clean metallic sodium in absolute ethanol and evaporating to dryness under vacuum.

Preparation of Cholesterol Esters of Long-chain Fatty Acids. The preparation of cholesteryl palmitate is given as an example. Sodium ethylate (0.05 to 0.1 g) was mixed with 4.3 g (0.01 mole) of cholesteryl acetate and 3.0 g (0.01 mole) of methyl palmitate in a 200-ml round-bottomed ground-neck flask. The flask was connected to a manometer and aspirator through a trap cooled in an acetone-dry ice bath. Arrangement was also made by means of a T-tube and clamp to introduce pure nitrogen in the flask when desired. The flask was flushed several times with nitrogen and then heated under vacuum (20 to 30 mm) at 80° to 90° for 1 hour with occasional gentle shaking to mix the contents. The evolution of methyl acetate was observed by the effervescence, which gradually subsided toward the end of the reaction. The reaction mixture was cooled and washed with two 100-ml portions of petroleum ether (b.p. 30°–60°) and filtered to remove insoluble material. The filtrate was evaporated to dryness under vacuum. The residue was refluxed twice with 50- to 75-ml portions of absolute ethyl alcohol, cooled to room temperature, and filtered. The filtrate was discarded and the residual cholesteryl palmitate was crystallized from acetone.

The cholesterol esters of other saturated fatty acids were prepared by the same procedure using appropriate quantities of their methyl esters. The cholesterol esters of lauric and myristic acids were crystallized from ethyl alcohol, those of palmitic and stearic from alcohol or acetone, and those of higher fatty acids from a mixture of ethyl ether and ethyl alcohol. The yields and physical properties of the sterol esters are listed in Table 1.

Purification of the Cholesterol Esters of Unsaturated Fatty Acids. The experimental conditions for the preparation of cholesterol esters of unsaturated fatty acids are essentially the same as those described for the preparation of the esters of saturated fatty acids. Because of their lower melting points and differences

TABLE 1. YIELDS AND PHYSICAL PROPERTIES OF CHOLESTEROL ESTERS OF LONG-CHAIN FATTY ACIDS

Ester	Yield (%)	Observed M.P. (°)*	Observed $[\alpha]_D^{25}$§	Literature M.P. (°)	Literature $[\alpha]_D$	Ref.
Laurate	81.5	91 –92	–30.6	78	–30.3	11
				91	–27.6	1
				100		12
				110	–31.3	13
Myristate	79	70 –70.5	–26.9	80 –86	–26.6	1
				73 –80	–26.5	3
Palmitate	77	77 –78	–24.8	77 –90	–25.1	1
				75 –80	–25.4	3
Stearate	81	81.5–82.5	–23.7	78 –82.5	–24.3	1
				71 –79.5	–25.1	3
Arachidate	87	85 –85.5	–23.2			
Behenate	85.5	87.5–88	–22.6			
Oleate	74	46.5–47	–24.5	44.5	–23.4	1
				45.0	–21.6	3
Linoleate	72	42	–24.7	42 –42.5	–23.9	4
				39 –40.3	–24.7	5
Linolenate	71	32 –33	–24.4	32.5–33.5	–24.3	5
Arachidonate†	69.5	24 –24.5	–23.4			
Arachidonate‡		22 –23	–23.2			

* M.P. uncorrected.
† Prepared from synthetic ethyl arachidonate.
‡ Prepared from methyl arachidonate of natural origin (hog liver).
§ Optical rotations were measured on 5% solutions in chloroform with a Bellingham & Stanley polarimeter.

in solubility, however, the purification procedures had to be modified. The petroleum ether solution of the reaction mixture was filtered and the filtrate evaporated to dryness under vacuum. The viscous residue was boiled for 2 to 3 minutes with two 25- to 50-ml portions of absolute ethyl alcohol, cooled to 0°, and the supernatant fluid decanted. The residue was dried under vacuum. Repetition of the same procedure or crystallization from organic solvents did not remove traces of the starting material, cholesteryl acetate. Hence, the sterol esters were purified by silicic acid column chromatography. Cholesteryl oleate, however, was obtained pure without recourse to chromatographic procedures. The purification of cholesteryl arachidonate prepared from synthetic ethyl arachidonate will be described in detail.

Silicic acid (Mallinckrodt 100 mesh, chromatographic grade) was prepared for chromatography as described by Hirsch and Ahrens (19) without grinding the commercial product. The apparatus was the same as described by them. Thirty grams of silicic acid was made into a slurry with petroleum ether (b.p. 60°–80°), poured in the column, and settled by frequent tapping with a glass rod. Approximately 2.5 g of the crude cholesteryl arachidonate dissolved in 10 ml of petroleum ether (b.p. 60°–80°) was applied on the column and washed with another 5 ml of the solvent. The solution was allowed to flow in without pressure until the liquid had been completely absorbed. The ester was then eluted with 1 liter of a mixture of petroleum ether (b.p. 60°–80°) and benzene, 80:20 (v/v) and collected in 25-ml fractions. Each fraction was monitored for the presence of impurities by TLC (vide infra). Most of the cholesteryl arachidonate was recovered by eluting with 650 ml of the solvent mixture. Only prolonged elution with petroleum ether-benzene eluted the cholesteryl acetate. The cholesteryl arachidonate was recovered by evaporation of the solvents under vacuum and was crystallized from acetone. It crystallized in clusters of needles from acetone on holding overnight at −20°. For purposes of comparison, a sample of cholesteryl arachidonate was also synthesized from methyl arachidonate prepared from hog liver. Since the methyl arachidonate from the natural source was only 90% to 95% pure as tested by gas-liquid chromatography, the cholesteryl arachidonate was subjected to fractionation by column chromatography and the fractions monitored by TLC. The fractions exhibiting more than one spot in the thin-layer chromatogram were discarded. The fractions containing only one component were combined and the cholesteryl arachidonate was recovered and crystallized as described above. The physical properties of this material closely resembled those of the cholesteryl arachidonate prepared from synthetic ethyl arachidonate and are shown in Table 1. The cholesterol esters of the other unsaturated fatty acids were purified by the same procedure. Cholesteryl oleate was crystallized from an alcoholic solution maintained at 5° for 1 hour and the linoleate and linolenate were crystallized from an acetone solution maintained overnight at −20°. The yields and physical properties of the cholesterol esters are shown in Table 1.

The usefulness of the present method for the preparation of labeled cholesterol esters of long-chain fatty acids in small quantities was also demonstrated by the preparation of C^{14}-labeled cholesteryl oleate from cholesteryl acetate and randomly labeled C^{14}-methyl oleate on a microscale.

Recovery of Fatty Acids as Their Methyl Esters from the Corresponding Cholesterol Esters. In order to confirm that no alterations in double bond structures occurred in the unsaturated fatty acid moiety of the sterol esters, methyl linolenate and arachidonate were prepared from 10-mg quantities of cholesterol esters as described by Stoffel *et al.* (20) and subjected to ultraviolet, infrared absorption, and gas-liquid chromatographic analyses. No detectable alteration in structure was noticeable by any method. The complete conversion of the cholesterol ester to the methyl ester was also ascertained by TLC.

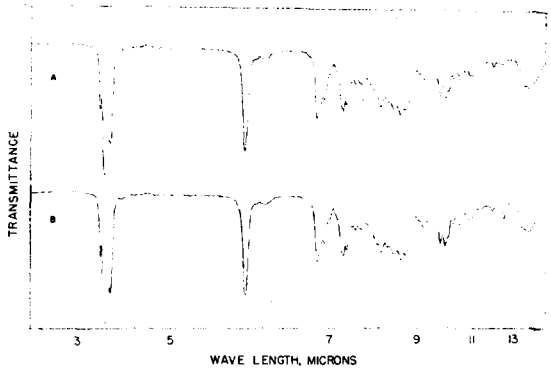

FIG. 1. Infrared spectra of cholesteryl arachidonate, prepared from (A) synthetic ethyl arachidonate and (B) methyl ester of arachidonate of natural origin (hog liver).

Infrared Analysis. The infrared spectra were obtained with a Perkin-Elmer Model 21 double-beam infrared spectrophotometer equipped with sodium chloride optics. Solution spectra were measured in a 0.1-mm cell, using 10% solutions in carbon disulfide (2.0 to 4.2 μ, 5 to 6.1 μ, and 7.2 to 15.0 μ), and in tetrachloroethylene (4.2 to 5.0 μ and 6.1 to 7.2 μ). The infrared spectra of cholesteryl arachidonate prepared from both synthetic and natural arachidonate are identical in all respects (Fig. 1).

Thin-layer Chromatography (TLC). Smooth glass plates, oblong (5 x 20 cm) and square (20 x 20 cm), were covered with a thin layer of Silica Gel G®[1] by spreading a mixture of 1 part of Silica Gel G® and 2 parts of distilled water with a thin-layer applier[2] as described by Stahl (21). The plates were dried at 105° for 6 hours. Five-microliter aliquots of a 1% solution of the cholesterol esters and cholesterol in petroleum ether (b.p. 60°–80°) were spotted 2 cm from one edge and 2 cm apart. The plates were developed by the ascending technique with a solvent mixture of petroleum ether (b.p. 60°–80°) and benzene, 60:40 (v/v) for 1 hour. The plates were then removed and the solvents were driven off in air. The plates were then sprayed with 50% sulfuric acid and heated over a hot plate for 5 to 10 minutes. Figure 2 shows the positions of cholesterol, cholesteryl acetate, and the cholesterol esters of the long-chain fatty acids. In working with cholesterol esters of labeled fatty acids, both chemical and radioactive assay methods should be used to obtain information about both chemical purity and radioactive purity. The thin-layer chromatogram of cholesteryl oleate-C^{14}, crystallized from alcohol, was found to be pure as tested by the sulfuric acid treatment and autoradiography.

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DISCUSSION

Several advantages may be cited in favor of the ester interchange method for the preparation of cholesterol esters of long-chain fatty acids. They are (*a*) the ready availability of the methyl esters of fatty acids from distillation procedures, (*b*) the elimination of costly and time-consuming procedures for conversion of the methyl esters to acids and then to acid chlorides or acid anhydrides, (*c*) the mild reaction conditions with high yields of product, (*d*) the absence of any alterations in double bond structures in polyunsaturated compounds, and (*e*) the ready applicability of the reaction to small-scale preparations of labeled compounds.

The purification of the cholesterol esters of saturated fatty acids and oleic acid presented few difficulties. They were all obtained pure by crystallization from common organic solvents. It was not feasible, however, to remove traces of the starting material, cholesteryl acetate, from the cholesterol esters of polyunsaturated fatty acids by repeated crystallizations. This does not detract from the usefulness of the method, as the cholesterol esters can be freed of the impurities by a simple column chromatographic procedure. In a petroleum ether-benzene mixture, the cholesterol esters of unsaturated acids migrate through a silicic acid column at a much faster rate than either cholesteryl acetate or cholesterol; hence the purification is readily effected. The cholesterol ester of arachidonic acid has been obtained in a crystalline form by this method.

The present method is highly suitable for preparing cholesterol esters of labeled fatty acids in small quantities. As an example, cholesteryl oleate has been prepared using randomly labeled C^{14}-methyl oleate.

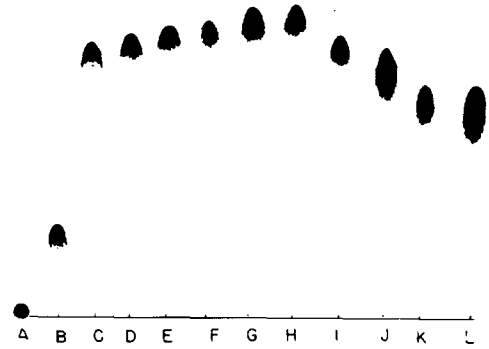

FIG. 2. Thin-layer chromatogram of cholesterol and cholesterol esters. Plate was eluted with a mixture of petroleum ether (b.p. 60°–80°) and benzene, 60:40 (v/v), the spots developed by charring after spraying with 50% sulfuric acid, and reproduction made by photo-copying. Samples were: A, cholesterol; B, cholesteryl-acetate; C, -laurate; D, -myristate; E, -palmitate; F, -stearate; G, -arachidate; H, -behenate; I, -oleate; J,-linoleate; K, -linolenate; L, -arachidonate.

The infrared spectra of the cholesterol esters of the saturated and the C_{18} unsaturated acids prepared by this method were identical with those described by Labarrère *et al.* (5). The infrared spectrum of cholesteryl arachidonate is reported here for the first time (Fig. 1). Labarrère *et al.* (5) used the spectra between 8.5 and 9.5 μ to differentiate the unsaturated esters from the saturated ones and also to differentiate the unsaturated esters from each other. The infrared spectrum of cholesteryl arachidonate in this region is found to differ from those of cholesterol esters of oleic, linoleic, and linolenic acids. The peak at 8.8 μ increases with increasing unsaturation in the C_{18} unsaturated esters but is virtually nonexistent in the cholesteryl arachidonate. Instead, a broader and more intense absorption appears at 8.65 μ and this is poorly resolved from the ester peak at 8.5 μ. Other variations are the absence of the 7.93-μ peak, the appearance of a new peak at 8.35 μ, and a shoulder at 8.2 μ. The infrared spectra of cholesteryl arachidonate prepared from arachidonate of both natural and synthetic origin are seen to be identical.

The fatty acid moieties from the cholesterol esters of polyunsaturated acids were recovered as their methyl esters without any change in configuration. No conjugation or *cis-trans* isomerization took place as observed by ultraviolet and infrared spectra.

The quick and convenient technique of TLC, developed by Stahl (21) and applied to the analysis of lipids by Mangold and Malins (22, 23), is used to advantage in detecting impurities such as cholesteryl acetate or free cholesterol in the sterol ester preparations. Detection and removal of free cholesterol from cholesterol esters is frequently accomplished by chemical means involving precipitation with digitonin. Kuksis and Beveridge (6) have shown that this method of separating free sterol from sterol esters is not very sensitive. Thin-layer chromatography affords a rapid method of detecting the presence of free cholesterol or cholesteryl acetate in microgram quantities since the cholesterol esters of long-chain fatty acids migrate at a much faster rate than cholesteryl acetate, while free cholesterol remains stationary. The rate of migration of cholesterol esters of saturated fatty acids increases with increasing chain length but these differences are not great enough to effect separation of the individual esters in a mixture. The migration rate of the cholesterol esters of unsaturated fatty acids possessing the same carbon chain length decreases with increasing unsaturation. Samples of cholesteryl linolenate and arachidonate obtained pure by column chromatography, as monitored by TLC, underwent oxidation on standing, and their oxidation products did not move on TLC.

Attempts to effect a clear-cut separation of the cholesterol esters of long-chain fatty acids from each other are in progress.

The authors are grateful to Dr. Jacques Chipault and George Mizuno for assistance in carrying out the determinations of infrared spectra.

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