Fatty Acid Specificity of Plasma Phosphatidylcholine:Cholesterol Acyltransferase*

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ABSTRACT: The fatty acid specificity of phosphatidylcholine: cholesterol acyltransferase (EC 2.3.1) was studied by assaying ultracentrifugal plasma preparations free of lipoproteins with sonicated dispersions of mixtures of unesterified cholesterol and phosphatidylcholine. Several phosphatidylcholines differing in their 2-position fatty acid were synthesized and served as substrates. The rate at which fatty acyl groups were transferred from the 2 position and subsequently esterified with the free cholesterol by the human plasma preparation was (in decreasing order): linoleate, oleate, arachidonate, positional isomers of oleic acid, trans isomers, and saturated fatty acids. The specificity was species dependent and remained uninfluenced by the nature of the fatty acid in the 1 position of phosphatidylcholine and by the relative molar proportions of phosphatidylcholine and free cholesterol in the incubation media.

Plasma phosphatidylcholine:cholesterol acyltransferase (EC 2.3.1) catalyzes the transfer of an acyl group from the 2 position of phosphatidylcholine to the hydroxyl group of cholesterol (Glomset *et al.*, 1962; Glomset, 1962; Shah *et al.*, 1964). The newly formed cholesterol esters appeared to be synthesized in amounts roughly proportional to the preexisting pattern of plasma cholesterol esters (Goodman, 1964; Portman and Sugano, 1964). This suggested that the enzyme possessed a fatty acid specificity characteristic of the normal pattern of plasma cholesterol esters of the particular species under study. Results with animals under abnormal diets, however, were not consistent with this conclusion and they pointed to the importance of the substrate composition, in particular, plasma lipoprotein structure and origin (Glomset, 1968).

The present paper describes work that we have done with enzymatic and substrate preparations essentially free of lipoproteins. This approach eliminated lipoprotein involvement and allowed a direct assessment of the enzymatic fatty acid specificity.

Materials and Methods

Cholesterol-*7a-t* from New England Nuclear Corp., Boston, Mass., was purified by argentation thin-layer chromatography (Morris, 1966) to a final purity better than 97%. It was diluted with carrier cholesterol to a specific activity of 10 mCi/mmole.

Palmitic-*1*-^{14}C, stearic-*1*-^{14}C, oleic-*1*-^{14}C, linoleic-*1*-^{14}C, oleic-*9,10-t*, and arachidonic-*5,6,8,9,11,12,14,15-t* acids were also purchased from New England Nuclear Corp.; elaidic-*1*-^{14}C, linelaidic-*1*-^{14}C, and palmitoleic-*1*-^{14}C acids from Applied Sciences Laboratories, State College, Pa. Each labeled fatty acid was purified by solvent partition (Borgstrom, 1952).

cis-Vaccenic-*11,12-t*, petroselenic-*6,7-t*, and *cis*-11-eicosenoic-*11,12-t* acids were synthesized, purified, and characterized, as previously described (Sgoutas, 1970).

The labeled acids were analyzed by gas–liquid radiochromatography. In each instance at least 96% of the radioactivity was contained in a single peak corresponding to the appropriate mass. The radioactive acids were diluted in the carrier fatty acids to specific activities ranging from 2 to 8 mCi per mmole. Unlabeled fatty acids were purchased from the Lipid Preparation Laboratory, Hormel Institute, Austin, Minn., and were rechecked for purity by gas–liquid partition chromatography.

1-Monoacylglycerophosphorylcholine was prepared from egg lecithin by treatment with the venom of *Crotalus adamanteus* (Moore and Williams, 1964). It was also prepared from dipalmitoyl- or dioleoylglycerophosphorylcholine (from Supelco, Inc., Bellefonte, Pa.). In each case, it was purified by silicic acid chromatography and recrystallized from hot ethanol.

For the introduction of the labeled fatty acid into the 2 position of 1-monoacylglycerophosphorylcholine, a modification of the biosynthetic procedure of Robertson and Lands (1962) was used. In a typical example, microsomal fraction from 2 g of rat liver was incubated in the presence of 50 μmoles of 1-monoacylglycerophosphorylcholine, 35 μmoles of oleic-*1*-^{14}C acid (2 mCi/mmole), 200 μmoles of ATP, 4 μmoles of CoA, and 200 μmoles of $MgCl_2$. The mixture was taken in 25 ml of sodium phosphate buffer (0.1 M, pH 7.5), the pH adjusted to 7.5, and the mixture was shaken for 3 hr at 37°. The product was recovered and purified as previously described (Robertson and Lands, 1962). By virtue of the positional specificity of phospholipase A_2 from *C. adamanteus* venom, 95% of the oleic-*1*-^{14}C acid was found to be located at the 2 position. The free fatty acids thus obtained were also analyzed by gas–liquid partition chromatography: oleic acid, 82.4%; linoleic, 9.5%; arachidonic, 6.9%; and palmitic, palmitoleic, and stearic acids were also present. In all instances the incorporated fatty acid comprised 75–85% of the total mass of the fatty acids in the 2-position of the synthetic phosphatidylcholine. The rest of the acids had their origin in microsomal phosphatidylcholine.

Whole blood was drawn from healthy subjects and was collected in Na_2EDTA (1 mg/ml). Freshly prepared plasma was raised to a salt background density of 1.21 g/ml by addi-

* From the Department of Pathology, Emory University School of Medicine, Atlanta, Georgia 30322. *Received September 7, 1971.* This work was supported by Grant HE 13980, from the National Institutes of Health, U. S. Public Health Service.

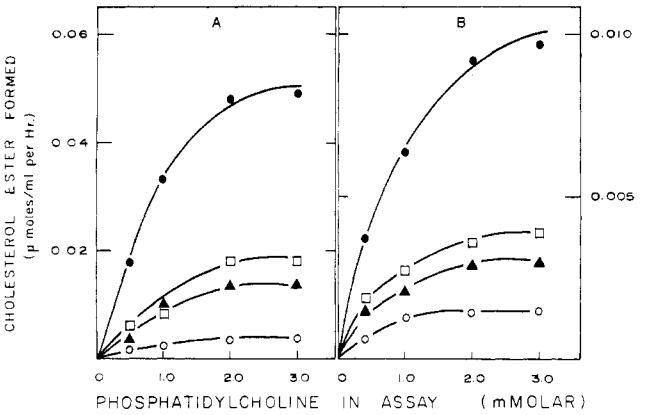

FIGURE 1: Esterification of free cholesterol with varying amounts of radioactive phosphatidylcholines: 2-linoleoyl (●), 2-oleoyl (□), 2-arachidonoyl (△), and 2-palmitoyl (○). The assay mixture consisted of ultracentrifugal protein (38 mg/ml) fraction from human plasma plus sonicated unesterified cholesterol–phosphatidylcholine dispersion (3:1, v/v). In a total volume of 2 ml, the relative molar portion of phosphatidylcholine and unesterified cholesterol was always 6 in expt A and 2 in expt B. Incubation for 1 hr at 37°. Values are the mean of duplicate determinations from two experiments.

TABLE I: Composition of Cholesterol-*t* Esters Synthesized by Human Phosphatidylcholine:Cholesterol Acyltransferase.[a]

2-Acylphosphatidylcholine Added	Ester (%)	% Total of Esters Saturated	Δ1	Δ2	Δ4
None	1	10	20	54	16
Palmitoyl	10	75	5	15	5
Oleoyl	16	1	85	8	6
Linoleoyl	23	4	4	88	4
Arachidonoyl	16	2	8	10	80
Palmitoyl + oleoyl +Linoleoyl + arachidonoyl[b]	25	5	18	60	17

[a] The sonicated substrates consisting of 0.8 μmole of cholesterol-*t* and 4 μmoles of each phosphatidylcholine were incubated with ultracentrifugal protein fraction (1:3, v/v). In 2 ml, the final background solution consisted of 0.01 M phosphate–0.19 M NaCl buffer (pH 7.4). Incubation for 20 hr at 37°. [b] Each one in 2 μmoles.

tion of KBr. It was ultracentrifuged and then dialyzed as previously described (Nichols and Gong, 1971). No lipoprotein could be detected electrophoretically, and only small amounts of lipid were found either by phosphorus (Bartlett, 1959) determination of a chloroform extract of the enzymatic preparation or by iodine visualization of a chromatogram of the chloroform extract.

Rats and rabbits were from the departmental colony and were fed regular diets. Blood was collected in heparinized syringes by cardiac puncture. Enzymatic preparations by ultracentrifugation were made as described above. Protein was determined according to Gornall *et al.* (1949).

Substrates were mixtures of free cholesterol and phosphatidylcholine in the form of sonicated suspensions, prepared according to Nichols and Gong (1971).

The ultracentrifugal protein fraction and the substrate solutions were preincubated separately at 37° prior to mixing. Aliquots of the protein fraction (1.5 ml) and the sonicated substrate (0.5 ml) were mixed and incubated at 37° with shaking. After appropriate intervals in incubation, the reaction was stopped with 25 volumes of chloroform–methanol (2:1, v/v). The lipid material was extracted (Bligh and Dyer, 1959) and separated by thin-layer chromatography on silica gel G. A two-step developing system was employed. Solvent I was chloroform–methanol–water (70:30:4, v/v) and it was allowed to run a distance of 10 cm from the origin. The plate was removed, dried under a stream of nitrogen for a few minutes, and then developed in solvent II which was hexane–diethyl ether (94:6, v/v) to 15 cm. After development, compounds were located with iodine vapor and the silica gel containing the compounds of interest was scraped from the plate and subsequently eluted (Kritchevsky and Malhotra, 1970). The eluents were taken in scintillation vials, the solvent was evaporated, scintillation solution added, and the radioactivity was measured.

Cholesterol-esterifying activity was expressed as disintegrations per minute per milliliter assay medium per hour of incubation after substracting the value for controls, containing 0.01 M *N*-ethylmaleimide. The number of micromoles of cholesterol esterified was determined from the specific activity of the phosphatidylcholine from the silica gel plate.

The distribution of radioactive cholesterol in different subclasses of cholesterol esters was determined on silica gel G impregnated with $AgNO_3$ as previously described (Morris, 1963; Goodman and Shiratori, 1964).

Results

In preliminary experiments, not shown, the extent of reaction of 2-linoleoyl-*1*-^{14}C-phosphatidylcholine with free cholesterol as a function of time when catalyzed with the ultracentrifugal protein fraction was linear for at least 40 min with less than 20% deviation from linearity in 1 hr. In addition, the reaction rate and enzyme concentration showed a linear relationship.

The fatty acid specificity was investigated in several ways. In one set of experiments (Figure 1) initial reaction rates were determined at varying concentrations of each of four different phosphatidylcholines. Among them, 2-linoleoylphosphatidylcholine had the fastest reaction rate followed by oleoyl-, arachidonoyl-, and palmitoylphosphatidylcholine, in decreasing order. In all cases, the rates increased with increasing substrate concentration until a plateau was reached at 2 mM concentration. At substrate concentrations higher than 4 mM the enzymatic activity was partially inhibited. With the relative proportions of phosphatidylcholine and free cholesterol in the substrate mixture changing from 6 to 2 (molar concentrations), approximately a fivefold decrease in reaction rates was observed (Figure 1A and B, respectively). Nevertheless, the overall pattern of specificity remained unchanged. Although it is not shown in these data, the specificity also remained unchanged when the substrate phosphatidylcholines were exclusively esterified with palmitic or oleic acid in the 1 position.

Table I presents data from analysis of cholesterol-*t* esters synthesized after addition of one or another phosphatidylcholine. Addition of 4 μmoles resulted in the predominant synthesis of the corresponding cholesterol ester, with 75–80% of the ester-*t* found in the appropriate chromatographic zone. When equimolar amounts of each of four different phosphatidylcholines (Table I) were added to a single incubation

TABLE II: Rates of Acyl Transfer by Human Phosphatidylcholine:Cholesterol Acyltransferase.[a]

2-Acyl in Phosphatidylcholine	nmoles/hr per ml of Assay Medium	
	2 mM	3 mM
Linoleoyl	52	50
Palmitoleoyl	19	21
Oleoyl	15	13
Arachidonoyl	13	11
cis-Vaccenoyl	11	11
Petroselenoyl	6	6
cis-Δ^{11}-Eicosenoyl	6	5
Palmitoyl	5	5
Elaidoyl	2	3
Linolelaidoyl	2	2
Stearoyl	0.8	0.7

[a] Conditions of incubation are described in the text. Molar ratio of phosphatidylcholine and free cholesterol in the sonicated dispersions was 6. Incubations were conducted for 1 hr and values represent the mean of two experiments and are corrected for controls.

TABLE III: Ratio of Appearance in Cholesterol Esters of Substrate in Relation to a Reference Phosphatidylcholine.[a]

Expt	2-Acylphosphatidylcholine		^3H:^{14}C Ratio	
	Tested	Ref	Phosphatidylcholine	Cholesterol Ester
A	Oleoyl-^{14}C	Oleoyl-*t*	2.1	2.0
	Linoleoyl-^{14}C	Oleoyl-*t*	2.2	0.6
	Palmitoyl-^{14}C	Oleoyl-*t*	3.8	11.2
	Elaidoyl-^{14}C	Oleoyl-*t*	4.2	32.0
	Linolelaidoyl-^{14}C	Oleoyl-*t*	4.0	32.4
	Stearoyl-^{14}C	Oleoyl-*t*	1.1	19.2
B	Arachidonoyl-*t*	Oleoyl-^{14}C	2.8	2.4
	cis-Vaccenoyl-*t*	Oleoyl-^{14}C	3.2	2.6
	Petroselenoyl-*t*	Oleoyl-^{14}C	3.0	1.2
	cis-Δ^{11}-Eicosenoyl-*t*	Oleoyl-^{14}C	2.7	1.0

[a] Conditions of incubation are described in the test and in Table II. The values represent the average of duplicate observations of two experiments.

mixture, cholesteryl-*t* linoleate was formed predominantly. Table I also indicated that endogenous phosphatidylcholine did not contribute significantly to the pool.

In the second portion of this study, fatty acids differing in configuration, position of double bond and chain length were studied. Table II gives the relative acyl-transfer rates of these fatty acids when being esterified in the 2 position of phosphatidylcholine. Increasing the concentration from 2 to 3 mM did not change the reaction rate, indicating that the enzyme was sufficiently saturated. A marked difference between cis and trans isomers was observed as well as among positional isomers of cis monoenoic acids. The chain length, on the other hand, seemed to have a definite effect on the reaction rates.

In order to ascertain that reaction rates were measured under uniform conditions of enzymatic activity and substrate presentation, a reference substrate was used as an "internal standard." In several experiments, 2-oleoyl-*9,10-t*-phosphatidylcholine was simultaneously and in equimolar concentrations sonicated and incubated with phosphatidylcholine containing ^{14}C-labeled fatty acids (Table III, exp A) and 2-oleoyl-*1*-^{14}C-phosphatidylcholine with phosphatidylcholine substrates containing ^3H-labeled fatty acids (Table III, expt B). Both isotopes were measured simultaneously in subsequent samples and the results were expressed as the ratio ^3H:^{14}C. At equal concentrations between the substrate and the internal standard the ratio of the two isotopes recovered in cholesterol esters was recorded in Table III. The data indicated that the ratio of transesteristerified ^3H- and ^{14}C-labeled fatty acids closely resembled the ratio of their relative reaction rates as shown in Table II.

Table IV compares the results of the acyltransferase studies with human preparation to those with rat and rabbit. This table was included in order to show that phosphatidylcholine:cholesterol acyltransferase activities of different animal species have different specificities. The data expressed as ratios of ^3H:^{14}C indicated that for rat plasma the order of preference was arachidonate > linoleate > oleate >. For rabbit plasma the order was linoleate > arachidonate > oleate.

Discussion

Portman and Sugano (1964) demonstrated previously that the reaction preferentially produced cholesteryl linoleate and arachidonate in man and rat, respectively. In those experiments, however, plasma lipoproteins served as substrates and they were incorporated into the assay system either as common constituents of the test plasma itself, or were added as part of heat-inactivated plasma to the test plasma. In one instance, the incubation mixture contained substrate lipoproteins from human and rat plasma. Consequently, several interpretations were possible, including enzymatic specificity for one or the other acid, preferential enzymatic accessibility to a particular lipoprotein structure (containing either linoleoyl- or arachidonoylphosphatidylcholine), or a preferential enzymatic reaction with homologous lipoproteins. Formation of one or the other enzyme–lipoprotein complexes could have resulted in transfer of different amounts of either linoleate or arachidonate, the predominant fatty acids in human and rat plasma phosphatidylcholines, respectively.

TABLE IV: Species Differences in Specificity of Phosphatidylcholine:Cholesterol Acyltransferase.[a]

2-Acylphosphatidylcholine		^3H:^{14}C Ratio			
Substrate	Ref	Phosphatidylcholine	Cholesterol Esters Man	Rat	Rabbit
Arachidonoyl-*t*	Oleoyl-^{14}C	2.8	2.5	25.2	3.0
Oleoyl-*t*	Linoleoyl-^{14}C	2.2	0.7	0.6	0.4
Arachidonoyl-*t*	Linoleoyl-^{14}C	1.8	0.4	4.3	0.5

[a] Conditions of incubation are described in the text and Table II. The values represent the average of duplicate observations of two experiments.

The present results clearly demonstrate that phosphatidylcholine:cholesterol acyltransferase distinguishes between different fatty acids in the 2 position of phosphatidylcholine. Thus, it permits unequivocal characterization of enzymatic specificity for certain fatty acids, in different animal species, by eliminating lipoprotein involvement. Furthermore, it indicates that although the activity changes in magnitude, the specificity is uninfluenced by changes in the relative proportions of phosphatidylcholine and free cholesterol in the sonicated mixtures. Considering that the relative content of phospholipid and free cholesterol varies in plasma lipoproteins of different density, and that the two lipids, either in sonicated mixtures or in plasma lipoproteins, are accessible to the enzyme in a comparable manner (Nichols and Gong, 1971), one can speculate that the specificity would not be dependent upon the density of lipoproteins. However, this hypothesis must be tested using actual lipoproteins as the substrates.

An assessment of the importance of specificity in determining the fatty acid composition of newly formed cholesterol esters can be made by comparing the data in Tables I and II. Under equal concentrations of phosphatidylcholines differing in their 2-position fatty acid (Table I), cholesterol esters are formed in proportions reflecting the relative reaction rates (Table II) of the corresponding fatty acids in the 2 position. This pattern represents the actual specificity of the enzyme and does not agree with the results of Glomset (1968). He reported that the relative distribution of radioactivity in cholesterol esters formed in human plasma was 12.3% for the saturated, and 20.6, 56.6, and 10.5% for the mono-, di-, and tetraunsaturated esters, respectively. The latter results, however, were influenced by the composition of the phosphatidylcholines from the plasma lipoproteins and in addition some of the fatty acids, especially saturated and monounsaturated, were undoubtedly transferred from nonphospholipid esters, such as glycerides (Shah *et al.*, 1964).

On the other hand, it is not possible from the available information (Glomset, 1968) to decide whether the specificity derives from fixed proportions of different acyltransferases with different specificities or from a single enzyme. Further purification and characterization of the enzymatic activity will be necessary before questions like this can be answered.

With regard to the molecular basis for such a specificity, it is possible that the physical state of the phospholipid may play a role. It has been shown already that this is the case for phospholipase A (Dawson, 1964). More likely, however, it can be attributed to a structural specificity of the enzyme or an activator peptide:phosphatidylcholine linked to protein by electrostatic bonds may confer acyl chain specificity through its hydrophobic residues.

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