Comparative studies on the substrate specificity of lecithin:cholesterol acyltransferase towards the molecular species of phosphatidylcholine in the plasma of 14 vertebrates

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Abstract Comparative studies indicate that plasma cholesteryl ester (CE) composition is correlated with susceptibility to atherosclerosis. We previously showed that the lecithin:cholesterol acyltransferases (LCATs) of susceptible species such as rabbit, pig, and chicken (group I) differ in their substrate and positional specificities from the LCATs of resistant species such as rat and mouse (group II). However, the relative importance of enzyme specificity and substrate phosphatidylcholine (PC) composition in determining the CE composition is not known. To address this, we analyzed the molecular species composition of plasma PC in the same 14 vertebrates in which we previously studied the CE composition and LCAT specificity. The utilization of native PC species by LCAT was studied by determining the loss of each PC after incubation of plasma at 37°C. The major contributor for LCAT reaction was either 16:0–18:2 PC or 18:0–18:2 PC in all species except dog, in which it was 18:0–20:4 PC. The formation of 20:4 CE correlated more with the consumption of 18:0–20:4 PC in group I, and with the consumption of 16:0–20:4 PC in group II. The group II enzymes exhibited higher selectivity for *sn*-2–20:4 PCs, whereas the group I enzymes showed preference for *sn*-2-18:2 PCs. The synthesis of high percentage of 20:4 CE in dog plasma was found to be due to the presence of unusually high concentration of 18:0–20:4 PC, rather than due to enzyme selectivity. These results show that the PC molecular species composition, especially the concentrations of *sn*-2-20:4 phosphatidylcholines has profound influence on plasma CE composition, and possibly on atherogenic risk.—**Subbaiah, P. V., and M. Liu.** Comparative studies on the substrate specificity of lecithin:cholesterol acyltransferase towards the molecular species of phosphatidylcholine in the plasma of 14 vertebrates. *J. Lipid Res.* 1996. **37:** 113–122.

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Phosphatidylcholine (PC) is the major phospholipid in the plasma of all vertebrates, and it serves not only as a structural component of lipoproteins and cell membranes but also as a substrate for several enzymes in plasma. Quantitatively, the most important enzyme reaction that utilizes PC is the lecithin:cholesterol acyltransferase (LCAT) activity. This reaction is the source of most of the cholesteryl esters (CE) in human plasma, although the tissue acyl CoA:cholesterol acyltransferase (ACAT) may contribute substantial percentage of plasma CE in other species (1, 2). The possible role of the fatty acid composition of CE, especially the low concentrations of 18:2 and 20:4, in influencing the atherogenic risk has been suggested by several studies (3–9), although some studies reported no such correlation (10, 11). Recent studies by Vesby et al. (12, 13) showed a strong negative correlation of the saturated CE concentration of serum with insulin sensitivity, which is another known cardiovascular risk factor, although it is not known whether the CE composition directly affects insulin sensitivity. Our recent results showed that animals known to be susceptible to atherosclerosis (e.g., rabbit, pig, guinea pig, chicken) have higher ratios of 16:0 CE/20:4 CE in their plasma, compared to the resistant species (e.g., rat, mouse) (14). Furthermore, we provided evidence that most of the differences in the CE composition between the resistant and susceptible species can be attributed to the differences in the substrate and positional specificities of their LCATs. Thus we found that isolated LCATs from the group I species, which were defined as those containing

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; CE, cholesteryl ester; PC, phosphatidylcholine; ACAT, acyl-CoA:cholesterol acyltransferase; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography.

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plasma 16:0 CE/20:4 CE ratios higher than that of human plasma, cannot utilize 16:0–20:4 PC efficiently, and when they do utilize it, their positional specificity is altered, resulting in the synthesis of more 16:0 CE than 20:4 CE. On the other hand, the LCATs isolated from group II species, which were defined as those containing 16:0 CE/20:4 CE ratios lower than human plasma, not only preferred 16:0–20:4 PC over other PCs, but also produced predominantly 20:4 CE in the presence of this PC. Majority of the species in group I (rabbit, pig, guinea pig, chicken, baboon, human, hamster, horse, sheep) are known to be susceptible to diet-induced atherosclerosis, whereas the majority of the species in group II (rat, mouse, cat, dog, cow) are known to be relatively resistant. Although the group I LCATs generated predominantly 16:0 CE in the presence of 16:0–20:4 PC, they produced mostly 20:4 CE in the presence of 18:0–20:4 PC, indicating that the molecular structure of PC, rather than its *sn*-2 acyl group alone, is important in determining the positional specificity of LCAT, and consequently the plasma CE composition. Therefore it is necessary to determine the molecular species composition of PC in the plasma and to investigate whether the utilization of individual PC species in native plasma differs in the resistant and susceptible species.

A comparative study on the substrate specificities of LCATs from various animal species has been previously carried out with isolated enzymes and synthetic symmetric PC substrates (15). The results obtained from this study, however, may not be applicable to native plasma because not only the composition of molecular species varies significantly among different animal species, but also the activator and inhibitor compositions are different. Furthermore, substrate specificity studies conducted with symmetric PC species, in which both positions of PC are occupied by the same fatty acid, cannot be compared to native plasma where most PCs are asymmetric, containing generally a saturated fatty acid at *sn*-1 position and an unsaturated fatty acid at *sn*-2.

In the present study we analyzed the molecular species composition of plasma PC in the same 14 vertebrate species in which we have previously studied the CE composition and synthesis (14). In addition, we studied the consumption of individual molecular species by LCAT reaction in native plasma in vitro to determine the specificity of LCAT towards the various PCs under physiological conditions. The results presented here show a wide variation not only in the molecular species composition of PC but also in the specificity of LCAT in native plasma. These results also show that in addition to the intrinsic specificity of LCAT, the plasma PC composition has significant effect on the CE composition and atherogenic risk.

MATERIALS AND METHODS

Plasma samples

Blood samples (drawn in 0.1% EDTA) from most animals were obtained from the Biological Resources Laboratories of University of Illinois at Chicago. Plasma samples from pigs were obtained from Dr. Jan Rapacz of University of Wisconsin at Madison, while the baboon plasma was supplied by Dr. Ram Kushwaha of Southwest Foundation for Biomedical Research, San Antonio, Texas. Human plasma was obtained from normal volunteers who had been fasting overnight. Pooled human plasma for the purification of LCAT was obtained from United Blood Services, Chicago. Frozen plasma samples from dogs and mice were purchased from Pel-freez Biologicals (Rogers, AR), for the purification of the respective LCATs. On the basis of the 16:0 CE/20:4 CE ratio in plasma, we have previously divided the various vertebrate species into two groups. Group I consisted of all species that have their plasma 16:0 CE/20:4 CE ratio equal to or higher than that of human plasma (horse, rabbit, guinea pig, pig, sheep, chicken, hamster, pig, baboon, human), while group II consisted of species whose 16:0 CE/20:4 CE ratio is lower than that of human plasma (cow, cat, dog, mouse, and rat) (14). This classification is used in the present work also. Furthermore, as the majority of the species in group I are known to be susceptible to atherosclerosis under experimental conditions as well as in nature, we have also empirically designated this group as "susceptible", and group II as "resistant".

Analysis of PC molecular species

Molecular species composition of plasma PC was analyzed by HPLC after conversion to diacylglycerol benzoates (16). The total lipid extract was first separated on silica gel TLC plates with the solvent system of chloroform–methanol–water 65:25:4 (v/v). The lipids were visualized by spraying the plate with 0.01% dichlorofluorescein in ethanol and viewing the plate under UV light. The spot corresponding to PC was scraped and eluted with chloroform–methanol (17). After taking an aliquot for phosphorus determination, the PC was hydrolyzed with phospholipase C (*Bacillus cereus*) (18), and the diacylglycerols formed were derivatized with benzoic anhydride as described by Blank et al. (16). The diacylglycerol benzoates were purified by TLC (18), and injected onto a C-18 column (Beckman Ultrasphere ODS) and separated with the solvent system of acetonitrile–isopropanol–water 70:27:5 (v/v) at a flow rate of 1.5 ml/min. The absorbance was monitored by an ISCO UV detector at 230 nm and quantitated on a Shimadzu CR3A integrator. The temperature of the column was

maintained at 30°C with the help of a column heater.

Determination of LCAT selectivity

Freshly prepared plasma samples were incubated at 37°C for 6 h without any addition. The consumption of individual molecular species of PC by LCAT was determined by the difference in the concentration of each PC at 0 h and 6 h. Total PC was determined by estimating lipid phosphorus (19) in an aliquot of PC before the phospholipase C treatment. The concentration of individual molecular species was calculated from the total PC concentration and the percentage of the molecular species was analyzed as diacylglycerol benzoates. The difference in the concentration of each PC species between 0-h and 6-h samples measures its consumption by LCAT. This value, when divided by the total decrease in PC, gives the percent contribution of individual PC for the LCAT reaction. The selectivity factor was calculated as the ratio of percent contribution by the percent concentration at 0 h.

Synthesis of labeled CE in vitro

To determine the effect of differences in PC composition on the synthesis of CE species by LCAT, we used heat-inactivated (58°C, 15 min) plasma from dog, mouse, and human as substrates and phenyl-Sepharose eluates from the plasma of the same species as the source of LCAT. Heat-treated plasma was first equilibrated with [4-^{14}C]cholesterol by incubation at 4°C for 18 h with cholesterol–albumin solution (20). Then the partially purified enzyme was added and the sample was incubated for 6 h at 37°C in the presence of 5 mM mercaptoethanol. The reactions were stopped by the addition of methanol, and the total lipids were extracted by the Bligh and Dyer procedure (17). The lipid extract was separated on a C-18 reverse phase column with the solvent system of acetonitrile–tetrahydrofuran–water 65:35:1.5 (v/v) at a flow rate of 2 ml/min and a temperature of 30°C. The radioactivity in the effluent was monitored with a Packard Radiomatic flow-through detector and quantitated using EZChrom data system (Scientific Software, San Ramon, CA).

Statistical analyses

All statistical analyses were performed with SPSS-PC software for Windows. Differences between various animal species were tested by one-way analysis of variance (ANOVA), with Tukey's honestly significant (HSD) post hoc test, and with significance level set at 0.05. Pearson correlation coefficients were used to measure the correlation between PC consumption and the formation of respective CE species.

RESULTS

Molecular species composition of PC

The total number of molecular species of PC detected in various animal plasma was about the same as we reported for human plasma (18, 21). The concentrations of 13 of the most abundant PC species are presented in **Table 1.** Smaller concentrations (0.01% to 1.01%) of 18:1–20:5 PC, 18:1–20:4 PC, and 18:0–20:5 PC were found in all plasma samples but are not included in the table. For ease of comparison, we kept the order of animal species in all tables and figures the same as in our earlier study, which was based on the decreasing order of 16:0 CE/20:4 CE ratio in the plasma. In contrast to the CE composition, there was no clear difference in the PC species composition between the atherosclerosis-susceptible (group I) and the atherosclerosis-resistant (group II) species. In general, the PC species containing 18:2 at *sn*-2 (16:0–18:2, 18:1–18:2, and 18:0–18:2) were the most prevalent in all animals, together constituting around 50% or more of the total PC in most plasma samples. The exceptions were dog plasma, which contained more *sn*-2–20:4 PCs than *sn*-2–18:2 PCs, and chicken plasma, which contained equal percentages of *sn*-2–18:1, *sn*-2–18:2, and *sn*-2–20:4 PCs. *Sn*-1–18:0 PCs were more abundant than the corresponding *sn*-1–16:0 species, especially when the *sn*-2 position is occupied by 18:2 or 20:4. However, when the *sn*-2 position is occupied by 18:1, the *sn*-1 contained more 16:0 than 18:0.

Among the individual PC species, 18:0–18:2 PC was the single most abundant PC in majority of the animals. It also showed the largest variation among various plasmas, ranging from less than 12% (human) to 50% (horse) of total PC. 16:0–18:2 PC was the predominant species in hamster, baboon, human, mouse, and rat, whereas dog and chicken had 18:0–20:4 PC as the major PC species. It is of interest to note that in most animal species (exceptions are human, hamster, and mouse) the concentration of 18:0–20:4 PC exceeded that of 16:0–20:4 PC. In view of our earlier finding that the positional specificity of some LCATs is altered in the presence of 16:0–20:4 PC but not in the presence of 18:0–20:4 PC (22), the relative ratios of these two PCs may affect the 16:0 CE/20:4 CE ratio significantly. Another PC that alters the positional specificity of LCAT, namely 16:0–22:6 PC (22, 23), was present in unusually high concentration in hamster plasma.

Consumption of individual PC species during LCAT reaction in intact plasma

As the predominant enzyme that breaks down native PC in normal plasma in vitro is LCAT, the decrease in various PC species after incubation of plasma can be

TABLE 1. Percent composition of molecular species of PC (mean ± SD)

Species (n)	16:0–20:5	16:0–22:6	16:0–20:4	18:1–18:2	16:0–18:2	16:0–20:3(n–6)
Horse (4)	0.11 ± 0.12^{a}	$3.18 \pm 0.79^{a,b,c}$	0.89 ± 0.13^{a}	7.87 ± 4.58^{d}	$25.16 \pm 5.48^{b,c,d,e}$	0.04 ± 0.03^{a}
Rabbit (5)	0.14 ± 0.15^{a}	$3.03 \pm 0.98^{a,b}$	2.61 ± 0.77^{a}	$3.73 \pm 0.74^{b,c}$	$26.41 \pm 2.41^{c,d,e}$	$0.61 \pm 0.61^{a,b}$
Guinea Pig (6)	0.11 ± 0.14^{a}	$3.24 \pm 1.57^{b,c}$	1.82 ± 0.52^{a}	0.73 ± 1.09^{a}	$21.98 \pm 1.94^{a,b,c,d}$	0.34 ± 0.75^{a}
Sheep (4)	$0.54 \pm 0.25^{a,b}$	$1.67 \pm 0.61^{a,b}$	$3.55 \pm 0.64^{a,b}$	$2.09 \pm 1.26^{a,b,c}$	$25.38 \pm 4.90^{b,c,d,e}$	$2.34 \pm 0.86^{c,d}$
Chicken (4)	$0.24 \pm 0.30^{a,b}$	$2.21 \pm 1.18^{a,b}$	$11.00 \pm 4.01^{d,e}$	$3.90 \pm 1.93^{b,c}$	13.56 ± 3.18^{a}	$1.11 \pm 0.67^{a,b,c,d}$
Hamster (4)	1.04 ± 0.17^{b}	15.79 ± 2.38^{e}	$11.42 \pm 2.03^{d,e}$	$0.97 \pm 0.10^{a,b}$	$32.96 \pm 2.56^{e,f}$	0.22 ± 0.16^{a}
Pig (7)	$0.42 \pm 0.26^{a,b}$	$1.26 \pm 0.22^{a,b}$	$6.79 \pm 2.14^{b,c}$	3.85 ± 0.76^{c}	$18.84 \pm 1.72^{a,b,c}$	0.45 ± 0.22^{a}
Baboon (7)	$0.41 \pm 0.30^{a,b}$	5.76 ± 1.72^{d}	$8.49 \pm 1.90^{c,d}$	$0.89 \pm 0.25^{a,b}$	$29.00 \pm 3.63^{d,e}$	2.47 ± 1.26^{d}
Man (6)	$0.66 \pm 0.63^{a,b}$	$5.50 \pm 0.49^{c,d}$	13.16 ± 2.80^{e}	$2.70 \pm 0.70^{a,b,c}$	$32.53 \pm 2.71^{e,f}$	4.57 ± 0.61^{e}
Cow (4)	0.12 ± 0.08^{a}	0.57 ± 0.20^{a}	$3.42 \pm 0.80^{a,b}$	$1.07 \pm 0.61^{a,b}$	$16.84 \pm 6.05^{a,b}$	$2.09 \pm 1.47^{b,c,d}$
Cat (4)	0.10 ± 0.10^{a}	$2.96 \pm 1.05^{a,b}$	$4.60 \pm 1.19^{a,b,c}$	$5.12 \pm 1.12^{c,d}$	$19.58 \pm 2.25^{a,b,c}$	0.05 ± 0.03^{a}
Dog (7)	0.17 ± 0.23^{a}	$1.28 \pm 0.59^{a,b}$	14.86 ± 1.94^{e}	$1.31 \pm 0.63^{a,b}$	15.10 ± 3.85^{a}	0.23 ± 0.16^{a}
Mouse (4)	1.06 ± 1.04^{b}	6.64 ± 0.59^{d}	$4.85 \pm 0.52^{a,b,c}$	$1.33 \pm 0.98^{a,b}$	40.80 ± 8.89^{f}	$0.88 \pm 0.54^{a,b,c}$
Rat (5)	0.98 ± 0.25^{b}	7.61 ± 0.73^{d}	$12.11 \pm 1.54^{d,e}$	$2.74 \pm 1.24^{a,b,c}$	$29.26 \pm 1.02^{d,e,f}$	0.40 ± 0.67^{a}

	16:0–20:3(n–9)	18:0–20:4	18:1–18:1	16:0–18:1	18:0–18:2	16:0–16:0	18:0–18:1
Horse (4)	$0.07 \pm 0.02^{a,b}$	2.03 ± 0.34^{a}	0.18 ± 0.24^{a}	4.50 ± 4.03^{a}	50.40 ± 6.42^{e}	$0.79 \pm 0.90^{a,b}$	$2.80 \pm 0.87^{a,b,c}$
Rabbit (5)	$1.02 \pm 0.41^{b,c,d}$	4.65 ± 1.65^{a}	$1.33 \pm 0.86^{a,b,c}$	$13.28 \pm 1.32^{b,c,d,e,f}$	$31.58 \pm 2.20^{d,e}$	$1.16 \pm 0.62^{a,b}$	$6.05 \pm 1.27^{b,c,d}$
Guinea pig	$0.16 \pm 0.18^{a,b}$	5.45 ± 1.03^{a}	0.34 ± 0.47^{a}	$7.61 \pm .65^{a,b,c}$	47.36 ± 1.97^{e}	$0.44 \pm 0.40^{a,b}$	8.29 ± 1.45^{d}
Sheep (4)	$1.34 \pm 0.55^{c,d,e}$	$8.68 \pm 1.15^{a,b}$	$0.53 \pm 0.36^{a,b}$	$9.23 \pm 4.06^{a,b,c,d,e}$	31.53 ± 3.31^{d}	$0.60 \pm 0.52^{a,b}$	9.84 ± 3.37^{d}
Chicken (4)	$0.97 \pm 0.89^{a,b,c,d}$	18.77 ± 6.36^{c}	$1.34 \pm 0.72^{a,b,c}$	17.86 ± 6.14^{f}	13.73 ± 3.73^{a}	$1.95 \pm 1.13^{a,b}$	$7.32 \pm 3.09^{c,d}$
Hamster (4)	0.03 ± 0.04^{a}	$7.36 \pm 1.26^{a,b}$	$0.37 \pm 0.34^{a,b}$	$9.04 \pm 0.93^{a,b,c,d}$	13.66 ± 2.77^{a}	0.07 ± 0.07^{a}	$1.97 \pm 0.85^{a,b}$
Pig (7)	$0.64 \pm 0.27^{a,b,c,d}$	$13.72 \pm 3.58^{b,c}$	2.17 ± 0.10^{c}	$14.76 \pm 2.59^{e,f}$	$24.66 \pm 4.75^{b,c,d}$	$1.92 \pm 0.29^{a,b}$	8.21 ± 0.35^{d}
Baboon (7)	$0.51 \pm 0.46^{a,b,c}$	$9.54 \pm 3.41^{a,b}$	$0.78 \pm 0.41^{a,b}$	$8.34 \pm 1.07^{a,b,c,d}$	$26.11 \pm 4.33^{b,c,d}$	$1.72 \pm 1.12^{a,b}$	$3.27 \pm 1.20^{a,b,c}$
Man (6)	$0.58 \pm 0.71^{a,b,c}$	5.89 ± 1.04^{a}	$0.63 \pm 0.55^{a,b}$	$14.27 \pm 3.38^{d,e,f}$	11.87 ± 3.20^{a}	$1.62 \pm 0.37^{a,b}$	$1.99 \pm 0.35^{a,b}$
Cow (4)	1.77 ± 0.51^{e}	$9.68 \pm 0.52^{a,b,c}$	$1.44 \pm 0.65^{a,b,c}$	$9.39 \pm 7.88^{a,b,c,d,e}$	$28.46 \pm 12.74^{b,c,d}$	8.23 ± 2.15^{c}	12.25 ± 5.88^{f}
Cat (4)	$0.08 \pm 0.07^{a,b}$	$9.66 \pm 4.11^{a,b,c}$	1.79 ± 0.72^{c}	$13.49 \pm 1.93^{b,c,d,e,f}$	$29.43 \pm 4.32^{c,d}$	$1.13 \pm 1.15^{a,b}$	8.90 ± 0.52^{d}
Dog (7)	$1.40 \pm 0.41^{d,e}$	34.36 ± 7.83^{d}	$1.44 \pm 0.47^{b,c}$	$6.89 \pm 1.02^{a,b}$	14.19 ± 4.08^{a}	2.15 ± 1.12^{b}	$3.72 \pm .50^{a,b,c}$
Mouse (4)	$0.06 \pm 0.05^{a,b}$	4.13 ± 1.75^{a}	$0.58 \pm 0.68^{a,b}$	$14.17 \pm 4.78^{c,d,e,f}$	$19.69 \pm 4.28^{a,b,c}$	$0.92 \pm 1.02^{a,b}$	$2.40 \pm 1.05^{a,b}$
Rat (5)	$0.32 \pm 0.10^{a,b}$	$14.00 \pm 5.00^{b,c}$	$0.89 \pm 0.76^{a,b}$	5.57 ± 1.54^{a}	$17.43 \pm 2.28^{a,b}$	$0.91 \pm 0.58^{a,b}$	1.61 ± 0.65^{a}

Values without a common superscript in the same column are significantly different from each other by one way ANOVA, with Tukey-HSD post-hoc test, significance level, 0.05. Smaller percentages of 18:1–20:5 PC, 18:1–20:4 PC, and 18:0–20:5 PC were also detected in all plasma samples, but their concentrations exceeded 1% of the total in only one or two species, and therefore are not presented.

used to determine the physiological specificity of LCAT with respect to the PC species (18). We incubated freshly prepared plasma from each of the animal species at 37°C for 6 h and analyzed the molecular species composition of PC before and after incubation. From the differences in the concentrations of various PC species at 0 h and 6 h, we calculated the consumption of individual PC species by the LCAT reaction. The decrease of individual PC species as a percentage of total decrease in PC gives the percentage contribution of the particular PC towards LCAT reaction. **Table 2** shows the percentage contribution of all PC species to LCAT reaction in various plasma samples.

Quantitatively, the major contributor for LCAT reaction was either 16:0–18:2 PC or 18:0–18:2 PC in all animal species except dog, where the single highest contributor was 18:0–20:4 PC. The *sn*-2 18:2 PCs (16:0–18:2, 18:0–18:2, and 18:1–18:2) accounted for more than 60% of the total decrease in PC in all species except chicken, pig, dog, and rat. These results are consistent with the results of in vitro synthesis of CE in the native plasma, where the major CE formed was 18:2 CE in most animals (14). A significant consumption of 16:0–20:4 PC was evident in the plasmas of rat, dog, human, hamster, and chicken. It should be pointed out that of these LCATs, only the rat enzyme is known to produce exclusively 20:4 CE from 16:0–20:4 PC, while the rest of the enzymes synthesize relatively more 16:0 CE. The contribution of 18:0–20:4 PC was significantly high in rat, dog, and chicken, and in all these cases the product should be 20:4 CE, on the basis of our previous results (22). 16:0–18:1 PC, another quantitatively important PC species in all animals, contributed to LCAT reaction significantly (over 10%) only in chicken, human, rabbit, cat, mouse, and sheep.

The formation of labeled 20:4 CE in vitro (14) correlated positively with the consumption of *sn*-2–20:4 PCs (16:0–20:4 PC + 18:0–20:4 PC + 18:1–20:4 PC) in both groups of animals ($r = 0.46$, $P < 0.002$ for group I; $r = 0.29$, $P = 0.16$ for group II). The labeled CE formation in group I correlated better with the consumption of 18:0–20:4 PC ($r = 0.39$, $P = 0.008$) than with the consumption of 16:0–20:4 PC ($r = 0.33$, $P = 0.027$). In group II, however, the correlation was better with the consumption of 16:0–20:4 PC ($r = 0.53$, $P = 0.008$) than that of 18:0–20:4 PC ($r = 0.16$, $P = 0.46$). Furthermore, when examined individually, 7 out of 9 species in group I showed negative correlation between the consumption

TABLE 2. Percent contribution of individual PC species for LCAT reaction (mean ± SD)

Species (n)	16:0–20:5	16:0–22:6	16:0–20:4	18:1–18:2	16:0–18:2	16:0–20:3(n–6)
Horse (4)	0.03 ± 0.03[a]	0.93 ± 0.71[a]	0.42 ± 0.09[a]	10.12 ± 7.10[c]	43.21 ± 7.60[d,e,f]	0.01 ± 0.01[a]
Rabbit (5)	0.03 ± 0.04[a]	1.34 ± 0.90[a]	1.88 ± 1.24[a]	4.03 ± 1.12[a,b]	44.66 ± 3.10[d,e,f]	0.38 ± 0.50[a,b]
Guinea Pig (6)	0.04 ± 0.06[a]	1.73 ± 1.41[a]	1.24 ± 0.65[a]	0.81 ± 1.35[a]	33.58 ± 8.96[b,c,d]	0.28 ± 0.67[a,b]
Sheep (4)	0.29 ± 0.18[a]	0.76 ± 0.35[a]	2.80 ± 0.57[a,b]	1.88 ± 2.02[a,b]	38.62 ± 8.86[b,c,d,e,f]	1.00 ± 0.54[a,b,c]
Chicken (4)	0.04 ± 0.02[a]	0.78 ± 0.90[a]	9.57 ± 4.46[b,c]	4.41 ± 2.81[a,b]	25.31 ± 5.10[a,b]	0.57 ± 1.04[a,b,c]
Hamster (4)	0.90 ± 0.41[a]	5.61 ± 4.77[b,c]	9.21 ± 1.05[b]	1.50 ± 0.58[a,b]	66.37 ± 0.25[g]	0.05 ± 0.05[a,b]
Pig (7)	0.59 ± 1.35[a]	0.48 ± 0.21[a]	3.40 ± 1.47[a]	4.90 ± 0.99[b]	29.04 ± 1.86[a,b,c]	0.18 ± 0.12[a]
Baboon (7)	0.16 ± 0.12[a]	2.51 ± 1.86[a,b]	4.94 ± 2.58[a,b]	0.67 ± 0.27[a]	39.96 ± 7.35[c,d,e,f]	1.53 ± 1.29[a,b,c]
Man (6)	0.25 ± 0.21[a]	2.82 ± 0.39[a,b]	9.39 ± 4.26[b]	1.41 ± 0.69[a,b]	51.30 ± 5.40[f]	1.90 ± 0.98[b,c]
Cow (4)	0.13 ± 0.24[a]	0.19 ± 0.15[a]	3.67 ± 3.98[a,b]	1.40 ± 0.90[a,b]	26.40 ± 8.81[a,b]	2.37 ± 1.16[c]
Cat (4)	0.01 ± 0.01[a]	1.35 ± 1.98[a]	5.23 ± 2.76[a,b]	3.98 ± 1.70[a,b]	34.05 ± 6.05[b,c,d,e]	0.02 ± 0.02[a]
Dog (7)	0.09 ± 0.14[a]	1.01 ± 0.14[a]	15.83 ± 4.65[c,d]	1.25 ± 0.60[a,b]	20.21 ± 6.46[a]	0.06 ± 0.05[a]
Mouse (4)	0.72 ± 0.76[a]	8.15 ± 0.76[c]	7.23 ± 2.67[a,b]	1.02 ± 0.89[a,b]	49.06 ± 4.81[e,f]	0.40 ± 0.21[a,b,c]
Rat (5)	1.00 ± 0.45[a]	5.51 ± 3.40[b,c]	16.82 ± 4.41[d]	2.19 ± 1.43[a,b]	33.04 ± 5.92[b,c,d]	0.15 ± 0.24[a,b]

	16:0–20:3(n–9)	18:0–20:4	18:1–18:1	16:0–18:1	18:0–18:2	16:0–16:0	18:0–18:1
Horse (4)	0.01 ± 0.01[a]	0.73 ± 0.40[a]	0.03 ± 0.03[a]	0.91 ± 0.55[a]	42.91 ± 4.35[d,e]	0.26 ± 0.45[a]	0.33 ± 0.13[a]
Rabbit (5)	0.53 ± 0.38[a,b]	1.59 ± 0.88[a]	1.24 ± 1.00[b,c]	17.78 ± 2.43[d]	23.08 ± 0.90[a,b]	0.32 ± 0.24[a]	2.86 ± 0.77[a,b,c]
Guinea pig (6)	0.07 ± 0.11[a]	3.82 ± 1.69[a]	0.18 ± 0.28[a]	7.15 ± 2.31[a,b]	45.83 ± 5.60[e]	0.21 ± 0.25[a]	4.94 ± 4.19[a,b,c]
Sheep (4)	0.72 ± 0.28[a,b]	4.25 ± 0.76[a]	0.32 ± 0.24[a,b]	10.06 ± 6.56[b,c]	34.81 ± 11.81[b,c,d,e]	0.26 ± 0.33[a]	3.71 ± 1.94[a,b,c]
Chicken (4)	0.27 ± 0.20[a]	9.20 ± 5.97[a]	0.62 ± 0.57[a,b,c]	24.83 ± 5.42[d]	15.80 ± 6.84[a]	2.16 ± 1.94[a]	6.10 ± 5.63[b,c]
Hamster (4)	0.04 ± 0.06[a]	4.67 ± 2.52[a]	0.19 ± 0.18[a,b]	5.37 ± 1.21[a,b]	14.17 ± 6.75[a]	0.11 ± 0.18[a]	0.87 ± 1.13[a]
Pig (7)	0.21 ± 0.14[a]	5.57 ± 3.52[a]	1.42 ± 0.27[c]	8.10 ± 5.79[a,b]	39.30 ± 9.13[d,e]	0.87 ± 0.38[a]	5.66 ± 0.86[c]
Baboon (7)	0.31 ± 0.32[a]	4.91 ± 2.48[a]	0.52 ± 0.33[a,b,c]	8.78 ± 3.98[b]	32.20 ± 9.02[b,c,d]	0.94 ± 1.02[a]	2.35 ± 1.05[a,b,c]
Man (6)	0.21 ± 0.19[a]	2.60 ± 1.34[a]	0.19 ± 0.22[a]	18.32 ± 3.59[d]	11.47 ± 3.33[a]	0.45 ± 0.27[a]	0.80 ± 0.39[a]
Cow (4)	1.21 ± 0.93[b]	7.08 ± 0.43[a]	1.16 ± 1.18[a,b,c]	7.00 ± 10.84[a,b]	36.67 ± 14.55[c,d,e]	8.64 ± 4.53[b]	3.98 ± 0.53[a,b,c]
Cat (4)	0.09 ± 0.12[a]	7.02 ± 3.51[a]	0.54 ± 0.30[a,b,c]	15.38 ± 6.11[c,d]	27.77 ± 12.73[a,b,c]	0.56 ± 0.65[a]	3.75 ± 1.95[a,b,c]
Dog (7)	0.56 ± 0.49[a,b]	34.25 ± 10.43[c]	1.02 ± 0.56[a,b,c]	4.84 ± 2.54[a,b]	16.85 ± 4.92[a]	1.15 ± 0.58[a]	2.51 ± 1.31[a,b,c]
Mouse (4)	0.03 ± 0.03[a]	4.08 ± 1.03[a]	0.24 ± 0.20[a,b]	10.93 ± 2.84[b,c]	16.28 ± 4.55[a]	0.64 ± 0.75[a]	1.05 ± 0.74[a,b]
Rat (5)	0.15 ± 0.12[a]	20.01 ± 6.43[b]	0.40 ± 0.35[a,b]	4.43 ± 0.92[a,b]	13.65 ± 5.35[a]	0.38 ± 0.28[a]	0.91 ± 0.63[a]

Values without a common superscript in the same column are significantly different from each other by one way ANOVA, with Tukey-HSD post-hoc test, significance level, 0.05.

of 16:0–20:4 PC and the formation of 20:4 CE (exceptions were hamster and pig). On the other hand, the consumption of 18:0–20:4 PC correlated positively with 20:4 CE formation in 6 out of 9 species in the same group (exceptions were horse, rabbit, and baboon). These results suggest that the major contributor for 20:4 CE synthesis in group I animals is 18:0–20:4 PC, not 16:0–20:4 PC, whereas in group II animals the reverse is true.

Selectivity of LCATs

Although the percent contribution of different PC species shows the quantitative importance of various PCs as sources of acyl groups, these values are greatly affected by the concentration of the PCs in circulating plasma and do not necessarily reflect the substrate specificity of LCAT. To determine the specificity of the enzyme in native plasma, we calculated the selectivity factors for the major PC species, which were obtained by dividing the percent contribution of each PC with its concentration in freshly drawn plasma (18). A PC species with a selectivity factor of > 1.0 is therefore preferentially utilized by LCAT, whereas one with a selectivity factor of < 1.0 is a poorer substrate, compared to 'average' PC. For these calculations, it is assumed that all PC species in plasma are equally accessible for LCAT reaction, although this may not be true in all animal species because of possible differential distribution of various PC species on the 'substrate' and 'non-substrate' lipoproteins. It is further assumed, for the calculation purposes, that all the decrease in PC concentration during incubation is due to LCAT reaction alone.

Figure 1 shows the selectivity factors for the two major *sn*-2–18:2 PCs, namely 16:0–18:2 and 18:0–18:2 PCs. All LCATs showed selectivity of > 1.0 for 16:0–18:2 PC, indicating that it is a preferred substrate compared to 'average' PC. Group II LCATs tended to have lower selectivity values than group I LCATs. The selectivity factors for 18:0–18:2 PC, on the other hand, were variable, with some LCATs showing lower than 1.0 and others higher than 1.0. Especially noteworthy is the high selectivity of pig LCAT for 18:0–18:2 PC compared to the majority of LCATs. Compared to 16:0–18:2 PC, the selectivity of 18:0–18:2 PC was lower in all animals except pig, although the concentration of 18:0–18:2 PC is higher in most of the animals.

In **Figure 2** the selectivity factors for 16:0–18:1 and 18:0–18:1 PCs are shown. All LCATs exhibited low preference for 18:0–18:1 PC, with no statistically significant difference between any of the enzymes. On the other hand, the selectivity for 16:0–18:1 PC was varied,

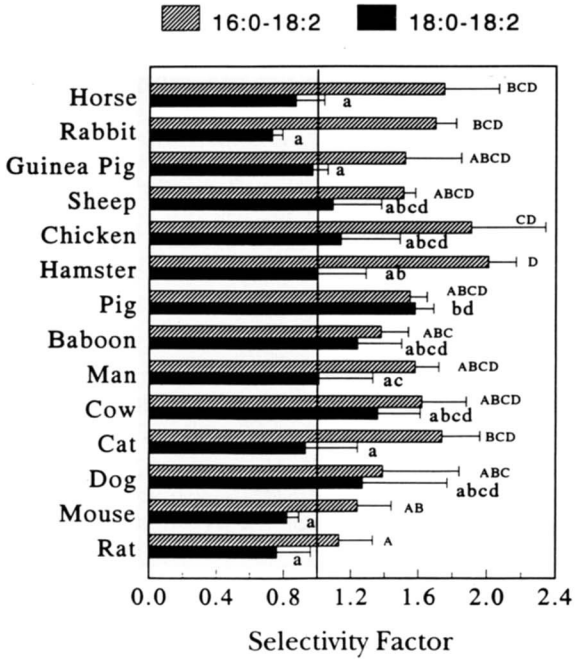

Fig. 1. Specificity of various LCATs for the major *sn*-2–18:2 PCs in native plasma. The selectivity factor for each PC was calculated by dividing its % contribution by its % concentration at 0 h. A selectivity factor of > 1.0 indicates that the enzyme prefers the particular PC over the "average" plasma PC, whereas a value < 1.0 indicates that it is a relatively poor substrate in native plasma. The error bar represents the standard deviation, and the number of samples for each animal species is the same as in Tables 1 and 2. The statistical significance of the difference in selectivity factors was determined by one-way ANOVA, using SPSS for Windows software. The bars without any common letters on top are significantly different from each other ($P < 0.05$). Note that upper case letters were used for 16:0–18:2 PC and lower case letters were used for 18:0–18:2 PC.

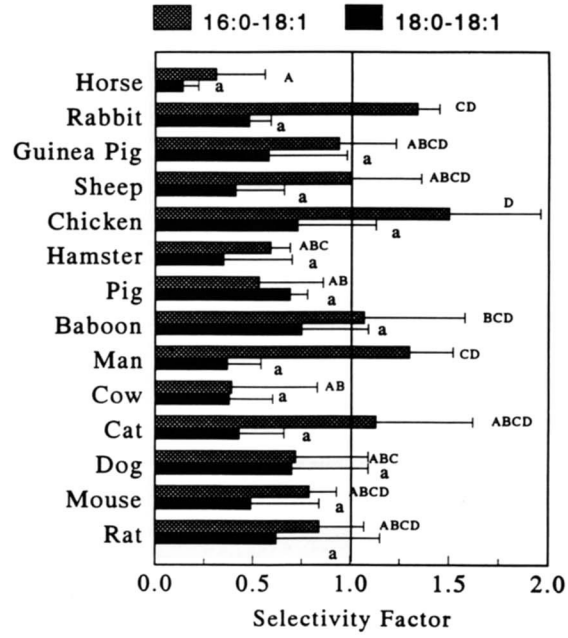

Fig. 2. Selectivity of various LCATs for the major *sn*-2–18:1 PC in native plasma. Bars without a common letter on the top are significantly different from each other as determined by ANOVA ($P < 0.05$).

with some LCATs (chicken, rabbit, human) showing higher selectivity, and others (horse, cow, pig) showing especially low selectivity. The low preference of horse and cow enzymes for 16:0–18:1 PC was also evident in our earlier experiments with isolated LCATs and synthetic substrates (14).

Figure 3 shows the selectivity values for the two major *sn*-2–20:4 PCs (16:0–20:4 and 18:0–20:4). Unlike other PC species, these PCs showed differences between the resistant and susceptible animals. Both PCs appear to be better substrates for group II enzymes (especially for mouse and rat) than for group I enzymes, as observed previously with isolated enzymes and synthetic substrate (14). All group I LCATs showed selectivity factors less than 1.0 for both the PCs, showing that these enzymes do not utilize the 20:4 acyl groups efficiently under native conditions. In all cases, except for rat and guinea pig enzymes, 16:0–20:4 PC was a better substrate than 18:0–20:4 PC.

The selectivity factors for the two major n–3 fatty acid-containing PCs are shown in **Fig. 4.** All LCATs except those of mouse and rat showed low selectivity for both the PCs. Similar results were obtained earlier with purified enzymes and synthetic PCs. (14). Although our previous results with purified human LCAT showed that

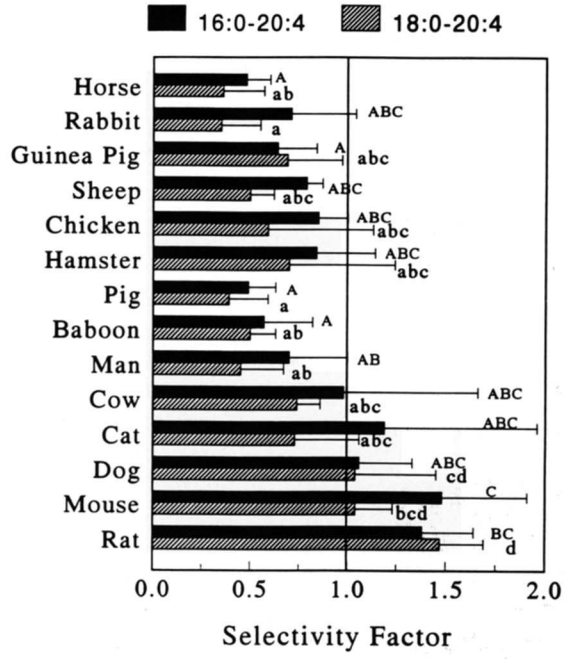

Fig. 3. Selectivity of various LCATs for the *sn*-2–20:4 PC in native plasma. Bars without a common letter on top are significantly different from each other ($P < 0.05$) by ANOVA.

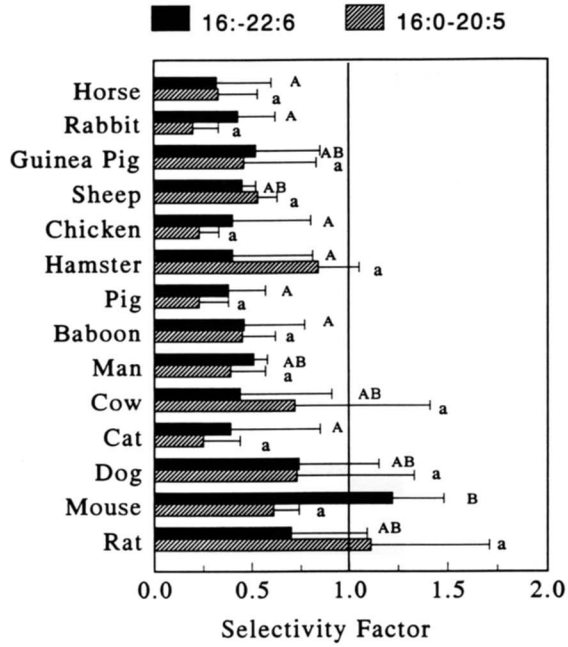

Fig. 4. Selectivity of various LCATs for 16:0–20:5 PC and 16:0–22:6 PC in native plasma. Bars without a common letter on top are significantly different from each other by one-way ANOVA ($P < 0.05$).

it preferred 16:0–20:5 PC over 16:0–22:6 PC by 2-fold (23), the results with native plasma show that 16:0–22:6 PC is utilized equally if not more efficiently than 16:0–20:5 PC. The enzymes from rat, hamster, cow, and sheep however, utilized 16:0–20:5 PC in preference to 16:0–22:6 PC. The difference between the in vitro and in vivo data may be due to differential distribution of the two PCs among various lipoproteins, or possibly to the difference in the activator specificities.

Effect of PC composition of plasma on the composition of CE species formed by LCAT: recombination experiments

Although we classified the dog as group II species based on the 16:0 CE/20:4 CE ratio in its plasma, the studies with isolated dog LCAT show that this enzyme behaves like a group I LCAT in the presence of 16:0–20:4 PC, producing more 16:0 CE than 20:4 CE (14). One possible explanation for this anomaly is the presence of a high concentration of 18:0–20:4 PC in dog plasma. The positional specificity of group I LCATs is not altered in the presence of 18:0–20:4 PC, and therefore the predominant CE formed is 20:4 CE, leading to low 16:0 CE/20:4 CE ratio in dog plasma compared to a plasma containing low concentration of 18:0–20:4 PC. To test this hypothesis, we used heat-treated dog plasma as the substrate and studied the formation of labeled CE from the added labeled cholesterol in the presence of purified human (Type I), and mouse (Type II) LCATs in addition to dog LCAT. We also performed the control experiments where the substrate used was the heat-treated human or mouse plasma instead of dog plasma.

As shown in **Table 3,** when human plasma was used as the source of substrates and activators, the compositions of labeled CE formed in the presence of human and dog LCATs were remarkably similar to each other. On the other hand, the labeled CE formed in the presence of mouse LCAT showed a distinctly different pattern, having a high percentage of polyunsaturated CE (20:5, 22:6, 20:4) and a low percentage of 16:0 CE and 18:1 CE. The results were essentially similar when mouse plasma was used as the substrate instead of human plasma. When heat-treated dog plasma was used as the substrate, however, both dog and human LCATs synthesized much higher percentage of 20:4 CE, compared with human or mouse plasma substrates. These results suggest that the high concentration of 18:0–20:4 PC in dog plasma results in the synthesis of high percentage of 20:4 CE even in the presence of group I LCAT. The percentage of 20:4 CE formed in dog plasma was even higher in the presence of mouse LCAT because of the additional effect of the selectivity of this LCAT for *sn*-2–20:4 PC species.

Figure 5 shows the 16:0/20:4 ratio in the CE newly synthesized by the three LCATs in the presence of

TABLE 3. Effect of plasma PC composition on LCAT specificity: recombination experiments

		Labeled CE species Formed (% of total)				
Substrate	LCAT	20:5 + 22:6	20:4	18:2	18:1	16:0
Human	Human	0.53	14.10	57.79	16.01	9.97
	Mouse	4.55	32.83	53.37	6.90	2.35
	Dog	0.51	14.35	57.05	18.68	9.42
Dog	Human	1.00	31.31	46.39	10.40	7.07
	Mouse	1.29	57.52	35.51	3.92	1.77
	Dog	1.40	31.47	46.52	12.87	7.75
Mouse	Human	2.08	14.23	56.52	18.15	9.01
	Mouse	1.07	44.87	41.13	8.43	4.50
	Dog	2.78	16.14	57.18	15.79	8.11

Heat-treated plasma (58°C, 15 min) from human, dog, or mouse was pre-labeled with [4-^{14}C]cholesterol and then incubated for 6 h at 37°C in the presence of 5mM mercaptoethanol and partially purified LCAT from human, dog, or mouse plasma. The labeled CE formed were separated on HPLC, and the radioactivity was determined in a flow-through radioactivity detector. The values given are averages of two separate experiments performed with two batches of plasma samples and enzymes. The ratios of 16:0 CE/20:4 CE are shown in Fig. 5.

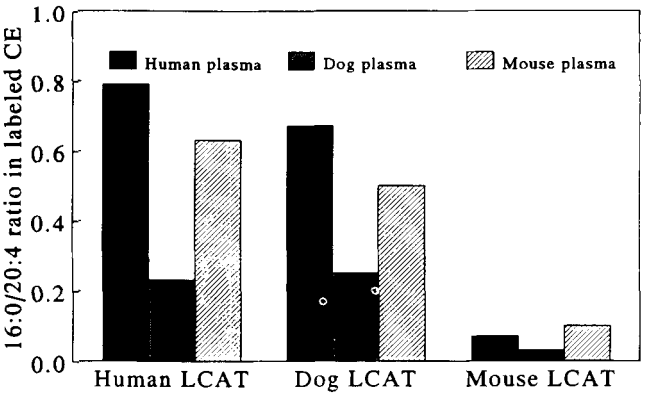

Fig. 5. Ratios of 16:0/20:4 in the newly formed CE by purified LCATs in the presence of heat-inactivated plasma as the source of substrate PC. The values are obtained from the data presented in Table 3, and are averages of two separate experiments.

various plasma substrates. While the mouse LCAT produced low 16:0/20:4 ratio in CE in the presence of all plasma substrates, the ratios resulting from the actions of dog and human LCATs were dependent upon the substrate used. The 16:0/20:4 ratios were significantly high when either human or mouse plasma was the substrate, and low when dog plasma was the substrate. These results therefore show that the composition of PC substrate, especially the high concentration of 18:0–20:4 PC, rather than the specificity of LCAT itself is responsible for the less atherogenic profile of CE composition in dog plasma.

DISCUSSION

The aim of the present investigation was to determine the relative importance of intrinsic LCAT specificity and plasma PC composition in influencing the plasma CE composition, and consequently the atherogenic risk. Although our previous results showed the importance of LCAT specificity in determining the CE species composition in various vertebrate species (14), the PC molecular species composition can have independent and significant effects on the overall CE composition of plasma. It is also known that the dietary fatty acid composition can have a direct effect on the molecular species composition of plasma PC and CE (24–26). Although we have obtained plasma samples from human volunteers and various animals consuming their habitual 'normal' diets, these diets may differ significantly from each other in their fatty acid composition and can thus influence the PC composition in plasma. In contrast to the data on plasma CE composition, however, there is very little information available on the molecular species composition of plasma PC in various vertebrate species, and the present study provides the first such comparative data. The only two species in which the PC molecular species composition of plasma has been previously analyzed in detail are rat (27, 28), and human (18, 28). Our data agree closely with the published values for these two species.

Unlike the CE composition, the PC molecular species composition did not clearly distinguish the atherosclerosis-resistant and atherosclerosis-susceptible species, indicating that the LCAT specificity is more important than the substrate PC composition in determining the overall CE composition. Nevertheless, several interesting differences were seen among various animal species. For example, the pure herbivores (horse, cow, rabbit, sheep) have low concentrations of 16:0–20:4 PC compared to the other animal species. However, the concentrations of 16:0–18:2 and 18:0–18:2 PCs were quite high in the same species, indicating a diminished capacity to convert 18:2 to 20:4 rather than dietary insufficiency of 18:2. It is of interest to note that humans on pure vegetarian diets also have been shown to have low concentrations of 20:4 in serum phospholipids and cholesteryl esters, although their 18:2 content is similar to that of omnivires (29). Although some previous studies reported that rat plasma contains over 50% of total PC as *sn*-2–20:4 PCs (1), our present results, as well as those of others (27, 28), show that only about 25% of the total PC in rat plasma is *sn*-2–20:4 PC. However, it has been shown by Koba et al. (30) that enrichment of diets with linoleic acid increases the concentration of *sn*-2–20:4 PC to 60% of total PC, indicating the importance of dietary fatty acid composition in determining the composition of plasma PC species, especially those containing essential fatty acids.

Most of the previous studies on the specificity of LCAT towards PC species were performed with isolated enzymes and synthetic substrates (15, 31, 32). However, these studies do not reveal the in vivo specificity of the enzymes because various PCs occur in widely varying concentrations and may be associated with different lipoprotein substrates that vary in their reactivity towards LCAT. Therefore, it is necessary to determine the utilization of different PC species by the enzyme in native lipoproteins. We have previously investigated the in vivo specificity of human LCAT by determining the consumption of individual PC species during LCAT reaction in native plasma (18). These results not only identified the major sources of acyl groups for CE formation but also the relative specificity of the enzyme under physiological conditions. We used this technique to determine the physiological substrates and the specificities of LCATs from various animal species. In order for the results to be valid, we have to make the assumption that all the decrease in the PC concentration is due to LCAT reaction alone. Although we cannot rule out the possibility of other lipolytic reactions during the

incubation of native plasma, it appears that this assumption is valid in most of the samples studied because the other plasma enzymes with known phospholipase A activities, namely the lipoprotein lipase and hepatic lipase, are released into circulation in active form only after intravenous injection of heparin (33). Furthermore, in most of the plasma samples studied, including human (34), cow (35), and horse (36), the decrease in PC during in vitro incubation has been shown to be equimolar to the synthesis of CE, showing that no phospholipase activity is present under these conditions. However, it has been reported that mouse plasma contains phospholipase A activity that is apparently due to hepatic lipase that is secreted even in the absence of heparin injection (37). If this enzyme is active in our samples, corrections may be required for the selectivity values of mouse LCAT.

Although the differences were not always statistically significant because of small sample size, group I LCATs exhibited higher selectivity for *sn*-2–18:2 PCs, and lower selectivity for *sn*-2–20:4 PCs when compared to group II LCATs. There were no apparent differences in the selectivity for *sn*-2–18:1 PCs between the two groups of LCAT. In general, the selectivity values support the conclusions drawn earlier (14) regarding the specificity of LCAT and atherogenic risk. Furthermore, the differences between the two groups appear to be due more to differences in LCAT specificity and less to variations in plasma PC composition. One exception for this was dog plasma, whose LCAT has the specificity of group I enzyme but whose CE composition is similar to that of group II plasma. This apparent discrepancy is due to the presence of unusually high concentration of 18:0–20:4 PC in dog plasma and high selectivity of dog LCAT for this PC. We previously showed that the positional specificity of human LCAT is determined by the chain length of the *sn*-2 acyl group (23). Although the positional specificity of all Type I LCATs is altered in the presence of 16:0–20:4 PC, resulting in the synthesis of more 16:0 CE than 20:4 CE, all LCATs synthesize more 20:4 CE than 18:0 CE in the presence of 18:0–20:4 PC (14). This is because the 20:4 acyl group is actually shorter than 18:0 (23) and is therefore transferred more efficiently than 18:0. Thus the utilization of relatively more 18:0–20:4 PC compared to 16:0–20:4 PC results in the less atherogenic CE profile even in the presence of a group I LCAT. The results of recombination experiments with heat-treated dog plasma and purified human LCAT further confirmed this hypothesis.

It is of interest to compare the results obtained here with native plasma with those obtained by Grove and Pownall (15) who used semi-purified LCATs and synthetic PCs. Based on the results with symmetric PCs, these authors concluded that human LCAT transferred saturated fatty acids more efficiently than unsaturated fatty acids and that it did not transfer either 20:4 or 22:6 to cholesterol. Our results, however, show that both 20:4 and 22:6 acyl groups are transferred by human LCAT as well as by other group I LCATs, and that 18:2 was transferred most efficiently by human LCAT. It should also be pointed out that significant amounts of 20:4 CE and 22:6 CE are synthesized in vitro by all plasma samples studied (14). We found that cow LCAT utilized *sn*-2–18:1 PCs poorly, when compared to *sn*-2–18:2 PCs (Fig. 1 and Fig. 2), whereas the results of Grove and Pownall (15) show that this enzyme transferred 18:1 and 18:2 equally efficiently from the symmetric PC substrates. The differences in the two studies is not only in the use of symmetric versus natural PCs but also in the use of native plasma substrates and activators versus reconstituted substrates. Nevertheless, it is necessary to take into account the structure of whole PC molecule rather than the *sn*-2 acyl group alone while studying the acyl specificity of LCAT.

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