Human plasma albumin transports [^{13}C]docosahexaenoic acid in two lipid forms to blood cells

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Abstract Docosahexaenoic acid (22:6) decreases blood platelet function and is highly concentrated in the brain where its depletion leads to functional impairments. Because the platelets and blood brain barrier capillary endothelium cannot hydrolyze the complex lipids for fatty acid (FA) uptake, nonesterified FA (NEFA) bound to albumin are assumed to be the delivery route of FA to these cells. The supply of ^{13}C-labeled 22:6 to blood cells by plasma albumin was studied in humans after a single ingestion of this FA esterified in a triglyceride (TG). The 22:6 ^{13}C/^{12}C ratio, measured by gas chromatography combustion-isotope ratio mass spectrometry was measured in lipid classes from albumin, platelets, leukocytes, and erythrocytes (taken as a tentative index of the brain uptake). Nonesterified [^{13}C]22:6 bound to albumin was rapidly produced after ingestion, as a result of the hydrolysis of very low density lipoprotein (VLDL) plus chylomicron TG. We found that albumin carried another source of 22:6, lyso-phosphatidylcholines (lyso-PC), in which [^{13}C]22:6 accumulated while the nonesterified [^{13}C]22:6 reached its minimal plasma concentrations. Computation of the relative contribution of NEFA and lyso-PC for the [^{13}C]22:6 delivery to platelets and erythrocytes showed that the [^{13}C]22:6 supply to platelets occurred uniquely through NEFA, whereas this pool was weakly involved in the delivery to erythrocytes. In contrast, lyso-PC was uniquely concerned with the 22:6 delivery to erythrocytes and represented the major part of this supply. We conclude that plasma albumin carries 22:6 in two lipid forms that are involved differently in the delivery of this FA to target cells.—**Brossard, N., M. Croset, S. Normand, J. Pousin, J. Lecerf, M. Laville, J. L. Tayot, and M. Lagarde.** Human plasma albumin transports [^{13}C]docosahexaenoic acid in two lipid forms to blood cells. *J. Lipid Res.* 1997. **38:** 1571–1582.

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Docosahexaenoic acid (22:6), a long chain polyunsaturated fatty acid (PUFA) from the n–3 family, is present at high levels in fish that also contain eicosapentaenoic acid, the precursor fatty acid. The decreased risk of cardiovascular diseases observed in populations consuming fish has been attributed to these two PUFAs (1, 2), although their relative importance in the effects observed and their specific properties are not fully elucidated (3). They exert antiatherogenic and antithrombotic effects through modulation of blood lipids and lipoproteins (4, 5). Given as a dietary supplement, they lower plasma triglycerides (TG), mainly by decreasing hepatic VLDL secretion and free fatty acid biosynthesis (6). Feeding 22:6-rich oil reduces the ratio of LDL to HDL, increases HDL_2, and decreases the LDL particle size and their cholesterol content (3, 7). The incorporation of long-chain n–3 PUFA in cell membranes also modifies eicosanoid production resulting in altered platelet and leukocyte reactivities (1, 8). Moreover, 22:6 is the sole n–3 PUFA that is highly concentrated in the brain (9) and retina (10), especially in neuromembranes such as brain synaptosomes and photoreceptor outer segments of the retina (11). Its depletion in these tissues during periods of growth leads to functional impairments in experimental animals (11) and infants (12), including abnormal electroretinogram, impaired visual acuity, and alterations in learning-related behav-

Abbreviations: BHT, butylated hydroxy toluene; GCC-IRMS, gas-chromatography combustion-isotope ratio mass spectrometry; CE, cholesteryl ester; 22:6, docosahexaenoic acid; HDL, high density lipoproteins; HPLC, high performance liquid chromatography; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; Lyso-PC, lysophosphatidylcholine; NEFA, nonesterified fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography; TG, triglyceride; VLDL, very low density lipoproteins; VLDL/Chyl, VLDL plus chylomicrons.

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iors. Although the biological properties of 22:6 have been well described, it is still unclear how this fatty acid is delivered to blood cells and to the brain. During its transport by chylomicrons and VLDL TG, 22:6 is released by lipoprotein lipase from capillary endothelium and utilized by tissues for lipid synthesis, storage, and oxidation. Concomitant to this hydrolysis, some 22:6 is made available to the blood and binds to albumin (13, 14). The fatty acid–albumin complex represents the generally admitted route by which fatty acids reach the brain (15), although other forms have been suggested (14, 16). For example, lyso-phosphatidylcholines (lyso-PC) are the second form of phospholipids (PL) in blood (17) and the injection of radiolabeled unsaturated lyso-PC induced better uptake of these fatty acids into the brain than the injection of unsaturated nonesterified fatty acids (18). Fatty acids bound to albumin are also substrates for inflammatory cell PL remodeling, in which the fatty acid released from the complex is activated into acyl-CoA and transesterified (19). Whereas this deacylation/reacylation pathway is a major route for incorporation of arachidonic acid into platelets (20) and leukocytes (21), there is no evidence that 22:6 is a substrate for this pathway in vivo. Moreover, if the 22:6 esterification into erythrocyte PL is taken as an index of 22:6 incorporation in the brain PL as it has been suggested (22), the chemical forms of 22:6 supplied to erythrocytes would reflect in a way those involved in the 22:6 uptake by the brain.

In the present study, the transport of 22:6 by albumin to target cells was studied in humans after a single ingestion of this fatty acid esterified in a TG and labeled with carbon 13 (^{13}C), a stable isotope of carbon. The 22:6 $^{13}C/^{12}C$ ratio, measured by gas-chromatography-combustion isotope ratio mass spectrometry (GCC-IRMS) is analogous to specific activity in radiotracer experiments and was used to establish the relationship between lipid compartments and to calculate the [^{13}C]22:6 amount accumulating in these pools over time. 22:6 was labeled in two lipid species bound to albumin: the nonesterified fatty acids (NEFA) and the lyso-PC. It is shown that the NEFA supply [^{13}C]22:6 mostly to platelets whereas lyso-PC delivers [^{13}C]22:6 uniquely to erythrocytes.

MATERIAL AND METHODS

Chemicals

D-[1-^{13}C]glucose was provided by Eurisotop (Saint Aubin, France). Docosahexaenoic acid (22:6), heptadecanoic acid (17:0) methyl ester, and internal standards for gas-chromatography (GC) analyses were purchased from Sigma Chemical (St. Louis, MO). Fatty acid standards for GC and high-performance liquid chromatography (HPLC) analyses were purchased from Nu-Chek Prep (Elysian, MN). Silica gel 60 plates and Superspher HPLC column were from Merck (Darmstadt, Germany). All solvents were analytical or HPLC grade and were from SDS (Peypin, France).

TABLE 1. Fatty acid composition of [^{13}C]22:6-triglycerides

Fatty Acids	mol %
10:0	0.8
12:0	2.4
14:0	13.6
16:0	21.6
16:1	1.3
18:0	0.6
18:1	15.0
22:6	44.4

Data are expressed as mol % of total fatty acids.

Synthesis of triglycerides containing [^{13}C]docosahexaenoic acid

These TG were produced by growing a microalgae strain (*Crypthecodinium cohnii*) in a defined medium (23) containing 3 g/l of D-[1-^{13}C]glucose. After harvesting the cells by centrifugation, the biomass was frozen-dried and total lipids were extracted with the isopropanol–hexane mixture 3:2 (v/v). TG purification was carried out on an alumina column, using hexane elution. [^{13}C]22:6n–3, represented 44 mol % of the total fatty acids (with less than 0.01 mol % of either 20:5n–3, 22:5n–3, or 20:4n–6) and had a ^{13}C abundance of 7.72 atom % (**Table 1**). Sixty percent of the [^{13}C]22:6 was located at the *sn*-2 position of the TG, as determined by the reaction with ethyl magnesium bromide (24). [^{13}C]22:6 methyl ester for the standard curve was prepared by transesterification of [^{13}C]22:6-TG with 5% H_2SO_4 in methanol, at 100°C.

Protocol

Three healthy male volunteers signed a written consent form after being informed about the purpose and modalities of the study. The scientific protocol was approved by the institutional human ethic committee. Subjects were instructed to maintain their usual diet, but excluding fish and marine foods, 1 week before and during the experiment, and alcohol 24 h before the protocol. Healthy subjects, fasted overnight, consumed 280 mg of tracer TG mixed in a yogurt (lipids:3.5%, proteins:3.7%, carbohydrates:5%) and had a breakfast immediately after this. At various times after ingestion, blood was taken by venipuncture into chilled plastic syringes containing ACD as anticoagulant (0.8% citric acid, 2% citrate, 2.45% dextrose, pH 4.5). A blood sam-

ple was also collected just before the tracer ingestion to measure the basal ^{13}C abundance in lipid pools.

Plasma and blood cell preparation

All experimental steps were performed at 4°C. Blood samples were transferred to plastic tubes kept on ice and immediately centrifuged at 100 *g* for 15 min to obtain platelet-rich plasma. This plasma was centrifuged at 1000 *g* for 10 min and the supernatant was treated for lipoprotein preparation. The resulting platelet pellet was rinsed twice and gently resuspended into a Tyrode/HEPES buffer (135 mM NaCl, 2.68 mM KCl, 0.46 mM $NaHPO_4$, 1 mM $MgCl_2$, 5 mM HEPES, 5.5 mM glucose, 1 mM EDTA, pH 6.4) (25). This cell suspension was immediately centrifuged at 1000 *g* for 10 min, rediluted into 4 ml of Tyrode/HEPES, without EDTA, at pH 7.35 and frozen at −20°C. For erythrocyte preparation, 10 vol of the pellet resulting from centrifugation of whole blood at 100 *g* was diluted into 10 vol of Tyrode/HEPES buffer and centrifuged at 100 *g* for 10 min. After removing the supernatant, the erythrocytes were diluted into 10 vol of 0.9% NaCl and centrifuged at 2000 *g* for 10 min. This procedure was repeated twice and 1 vol of Tyrode/HEPES containing butylated hydroxytoluene (BHT, 5×10^{-5}M) was added to the cells prior to freezing. For leukocyte preparation, 40 vol of 0.9% NaCl containing 1 mM of EDTA were added to 20 vol of blood cell pellet before mixing with 10 vol of 5% dextran 0.9% NaCl, 1 mM EDTA. After partitioning at room temperature, the supernatant containing leukocytes was centrifuged at 2000 *g* for 10 min. The supernatant was removed and the contaminating erythrocytes were eliminated by osmotic stress (26). The cells, rinsed by 2 vol of Tyrode/HEPES buffer, were frozen at −20°C, in the presence of BHT, as above.

Plasma albumin and lipoprotein separation

VLDL plus chylomicrons (VLDL/Chyl), HDL, and the plasma albumin fraction were prepared by ultracentrifugation at 600,000 *g* in a fixed-angle rotor (Kontron, TFT 80.49) for 2 h, using NaCl/KBr density gradient (27). The plasma density was increased to 1.31 g/ml by addition of 0.495 g of KBr/ml plasma. One ml of plasma was gently layered at the bottom of 2.4 ml NaCl/EDTA solution, the density of which was 1.006 g/ml (28).

Fractionation and quantification of lipids

Total lipids were extracted from blood cells and lipoprotein preparations according to Bligh and Dyer (29). VLDL/Chyl-TG were prepared by spotting the lipid extracts on silica gel 60 plates and eluting the TG (R_f: 0.58) with hexane–diethyl ether–acetic acid 80:20:1 (v/v/v) as the mobile phase. Lipids from HDL and albumin fraction were first separated into lyso-PC (R_f:0.20), phosphatidylcholine (PC) (R_f:0.47) and neutral lipids (R_f:0.91) on thin-layer chromatography (TLC) plates with chloroform–methanol–H_2O 65:25:4 (v/v) as the mobile phase. Neutral lipids were extracted from the silica gel with chloroform–methanol 90:10 (v/v) and were further fractionated into free fatty acids (R_f:0.24) and CE (R_f:0.94), by developing the TLC plate in hexane–diethyl ether–acetic acid 80:20:1 (v/v). Platelet, erythrocyte, and leukocyte PL classes were purified by TLC separation with chloroform–methanol–40% aqueous methylamine 60:20:5 (v/v) as the mobile phase (30), after predeveloping the plate with chloroform–methanol 80:8, (v/v). Lipid classes were scraped off the plate and treated with 5% H_2SO_4 in methanol for 90 min for fatty acid methyl ester preparation. Lipid classes were quantified by adding internal standards in the extraction mixture. Triheptadecanoylglycerol, 1,2-diheptadecanoylglycerophosphocholine, heptadecanoyllyso-PC, cholesteryl heptadecanoate, and 17:0 were added before lipid extraction, when appropriate, and the absolute amounts of fatty acid methyl esters were determined by GC analyses, relative to the known amount of added 17:0.

Gas-chromatography analyses

Fatty acid methyl esters were analyzed and quantified by GC, using a Delsi chromatograph model DI200 equipped with a Ross injector and an SP 2380 capillary column (30 m × 0.32 mm) (Supelco, Bellefonte, PA). The oven temperature was held at 145°C for 5 min and raised to 215°C at 2°C/min (31).

Purification of fatty acid methyl esters

Separation of fatty acid methyl esters was carried out by HPLC using a 5 μm Superspher 100 C18 column (4.6 mm internal diameter × 250 mm) and an isocratic elution with acetonitrile–H_2O 8:2 (v/v), at a flow rate of 2 ml/min (32). The fatty acid methyl esters were detected by UV absorbance at 210 nm. The 22:6 methyl ester was collected, rapidly taken to dryness under nitrogen, and redissolved into isooctane for GCC-IRMS analysis.

Gas-chromatography-combustion isotope ratio mass spectrometry analyses

Samples were injected into an HP 5890 gas chromatograph equipped with a Ross injector and a Supelco capillary column (SP-2380, 0.25 mm × 30 m). Temperature gradient was held at 160°C for 1 min and raised to 230°C at 20°C/min where it was maintained for 5 min. A heart-cut valve directed the helium flow either to the flame ionization detector or to a quartz tube filled with CuO, heated at 800°C, where CO_2 was generated from

fatty acids by catalytic combustion. The effluent was then driven into a water trap before its ionization by electron impact in the source of the isotope ratio mass spectrometer (SIRA 12, VG Isogas, Middlewich, UK). The different isotopomers were collected onto three different collectors at m/z 44 (main ion: $^{12}C^{16}O^{16}O$), 45 ($^{13}C^{16}O^{16}O$, $^{12}C^{16}O^{17}O$) and 46 ($^{12}C^{17}O^{17}$, $^{12}C^{16}O^{18}O$, $^{13}C^{16}O^{17}O$). Before and after the CO_2 peaks generated from the fatty acids, a CO_2 sample reference of known enrichment, calibrated against the international standard (Pee Dee Belemnite:PDB), was automatically injected into the mass spectrometer (28).

Expression of results

$^{13}C/^{12}C$ of samples and reference were obtained from the ratios between isotopomers 45 and 44, and used to calculate the δ per 1000 value ($\delta^{13}C‰$) with the following equation:

$$\delta^{13}C‰ = \frac{^{13}C/^{12}C \text{ sample} - {}^{13}C/^{12}C \text{ reference}}{^{13}C/^{12}C \text{ reference}} \times 10^3$$

where the reference is the international PDB standard, which is CO_2 obtained from the carbonate shell of a cretaceous mollusc.

From the $\delta^{13}C‰$, results were also calculated as atom % the atom % excess (APE) as described (13). The repeatability and precision of measurements were as previously reported (13, 28). The [^{13}C]22:6 appearance in biological samples was also expressed as the absolute amount of [^{13}C]22:6 in the lipid pools, at various time points, by determination of [^{13}C]22:6 dilution in the pools with a standard curve and by multiplying these values by the endogenous 22:6 concentration, quantitated by gas chromatography. The calibration curve was established by adding graded amounts of [^{13}C]22:6 enriched at +6450 $\delta^{13}C‰$, to commercial 22:6 (Sigma) at ^{13}C natural abundance. A linear regression line was calculated with the $\delta^{13}C‰$ values varying from −29.00‰ to +3000‰, as a function of the labeled molecule dilution. Area under the curve for the [^{13}C]22:6 concentration in VLDL/chyl-TG and NEFA bound to albumin were calculated according to the Romberg integration method (33) and were expressed as surface unit (SU). Differences between $\delta^{13}C‰$ values were detected by one-way ANOVA and differences between means were evaluated with Scheffé's test, using the STATVIEW SE program for Macintosh.

Calculations

A mathematical model based on the mass conservation principle has been carried out for determining the relative contribution of NEFA and lyso-PC to the [^{13}C]22:6 delivering to platelets and to erythrocytes. In what follows, the model is briefly presented. Let t_1 and t_2 be two time-dependent functions representing the total amounts of [^{13}C]22:6 carried by NEFA and by lyso-PC in blood, respectively. It is assumed that the total amounts of [^{13}C]22:6 in platelets and in erythrocytes are represented by time-dependent functions C_1 and C_2, respectively, the variations of which, with respect to time, verify:

$$\frac{d}{dt}C_1(t) = \alpha\gamma_1(t_1(t) - t_1^{as}) + \beta\gamma_2(t_2(t) - t_2^{as}) \quad \textit{Eq. 1)}$$

$$\frac{d}{dt}C_2(t) = (1-\alpha)\gamma_1(t_1(t) - t_1^{as}) + (1-\beta)\gamma_2(t_2(t) - t_2^{as}) \quad \textit{Eq. 2)}$$

Here α, β, γ_1, γ_2 are constants that represent the relative contribution of NEFA to platelets, the relative contribution of lyso-PC to platelets, and the fractions of NEFA and of lyso-PC involved in the process under our consideration, respectively. These constants are to be identified by means of experimental data. The constants t_1^{as} and t_2^{as} stand for the asymptotic values of functions t_1 and t_2 reached when the process does not evolve anymore. These constants are obtained from experimental data.

By integrating equations 1 and 2 from time t_i to time t_{i+1} the following expressions are obtained for functions C_1 and C_2:

$$C_1(t_i) - C_1(t_i + 1) = \alpha\gamma_1\int_{t_i}^{t_i+1}(t_1(s) - t_1^{as})\,ds + \beta\gamma_2\int_{t_i}^{t_i+1}(t_2(s) - t_2^{as})\,ds; \quad \textit{Eq. 3)}$$

$$C_2(t_i) - C_2(t_i + 1) = (1-\alpha)\gamma_1\int_{t_i}^{t_i+1}(t_1(s) - t_1^{as})\,ds + (1-\beta)\gamma_2\int_{t_i}^{t_i+1}(t_2(s) - t_2^{as})\,ds. \quad \textit{Eq. 4)}$$

By choosing the collection of times $\{t_i\}_{i=1}^{i=7}$ as the times where measurements take place ($t_1 = 2$; $t_2 = 4$; $t_3 = 6$; $t_4 = 12$; $t_5 = 24$; $t_6 = 48$; $t_7 = 72$) and by assuming that functions t_1 and t_2 vary linearly in $[t_i, t_{i+1}]$, numerical values for functions C_1 and C_2 depending on α, β, γ_1, γ_2 at times tj are obtained. If $\hat{C}_1(t_i)$, $\hat{C}_2(t_i)$ denote the measured values of total amounts of ^{13}C-22:6 in platelets and in erythrocytes, respectively, then the constants α, β, γ_1, γ_2 are calculated according to a least square minimizing procedure between these values and the

ones provided by numerical simulations (3)–(4):

$$\underset{\alpha,\beta,\gamma_1,\gamma_2}{\text{Inf}} \sum_{i=1}^{i=6} (\hat{C}_1(t_i) - (C_1(t_i))^2 + (\hat{C}_2(t_i) - C_2(t_i))^2$$

The computed values for parameters were $\alpha = 0.32$; $\beta = 0.0$; $\gamma_1 = 0.12$; $\gamma_2 = 1.0$.

RESULTS

The results in **Fig. 1** show that [^{13}C]22:6 appeared rapidly in the TG associated with a fraction containing VLDL and chylomicrons. Two hours after ingestion, the labeling was already maximal in VLDL/chyl-TG, with a δ^{13}C‰ value of +2725 ± 1204 δ^{13}C‰. A concomitant maximum of labeling was observed in NEFA bound to albumin, the [^{13}C]22:6 abundance in this fraction (+1709 ± 649 δ^{13}C‰) being 1.6-fold lower than in VLDL/chyl-TG. The [^{13}C]22:6 abundance decreased rapidly in both fractions between 2 and 12 h post-absorption to reach minimal plateau values 72 h after ingestion. At this time point, the 22:6 labeling was still high in both VLDL/chyl-TG (+88.0 ± 20.5 δ^{13}C‰) and NEFA bound to albumin (+38.9 ± 9.9 δ^{13}C‰). The [^{13}C]22:6 concentration in these two lipid classes was calculated from their δ_{13}C‰ values and from their endogenous 22:6 contents (Fig. 1b). Two hours after ingestion, the [^{13}C]22:6 amount in NEFA bound to albumin represented 11% of the amount present in VLDL/chyl-TG. From the area under the curve of [^{13}C]22:6 concentration in NEFA (562 SU) and VLDL/chyl-TG (7422 SU), it appeared that 7.6% of the [^{13}C]22:6 esterified in VLDL/chyl-TG was supplied to albumin, during the lipolysis.

Lipid forms of [^{13}C]22:6 associated with albumin

In this albumin fraction, two lipid species containing significant amounts of endogenous 22:6 were identified. The first one was NEFA in which the 22:6 concentration was 0.26 ± 0.08 μg/ml and the second one was the lyso-PC in which the 22:6 concentration was 0.32 ± 0.01 μg/ml, (mean ± SD, n = 3). The time course for the [^{13}C]22:6 appearance was somewhat different in NEFA and lyso-PC (Fig. 1a and **Fig. 2**). The labeling in NEFA was the highest at 2 h, and almost reached its minimal values by 6 h, at which time the labeling was maximal in lyso-PC. The 22:6 labeling was 20-fold lower in lyso-PC than in NEFA at 2 h, reached comparable levels at 12 h, and was 2.1-fold-higher 3 days after ingestion. From the δ^{13}C‰ values and endogenous 22:6 concentrations, the [^{13}C]22:6 accumulation over time was calculated in these two lipids (**Fig. 3**). Whereas the

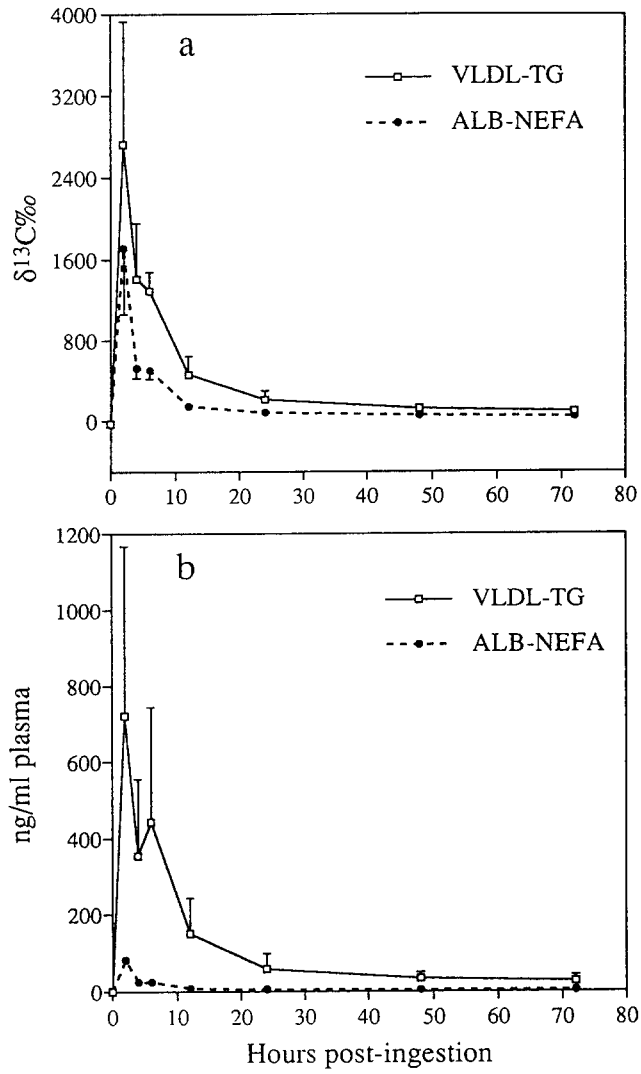

Fig. 1. Kinetics for the ^{13}C-22:6 abundance (δ^{13}C‰) in VLDL plus chylomicron triglycerides (VLDL-TG) and in nonesterified fatty acids bound to albumin (Alb-NEFA), after a single dose of ^{13}C-22:6-TG. Lipoproteins and albumin fraction were prepared by ultracentrifugation at 600,000 *g* for 2 h. Lipid classes were purified by TLC and fatty acid methyl esters were obtained by transmethylation. (a): the ^{13}C-22:6 abundance was measured by GCC-IRMS. (b): the ^{13}C-22:6 concentration in each lipid class was calculated from the endogenous 22:6 concentration, quantitated by GC. Each point represents the mean ± SD of values from three human subjects.

[^{13}C]22:6 concentration was 25-fold lower in lyso-PC than in NEFA at 2 h, it was higher after 24 h, reaching 1.7-fold at 72 h.

The origin of the [^{13}C]22:6-lyso-PC was further investigated by comparing the time course of [^{13}C]22:6 appearance into lyso-PC bound to albumin and into HDL-PC (**Fig. 4**). In human plasma, the prevalent lysolecithins are saturated and originate from the lecithin:cholesterol acyltransferase (LCAT) activity transesterifying the *sn*-2 fatty acid of HDL-PC to the hydroxyl group of

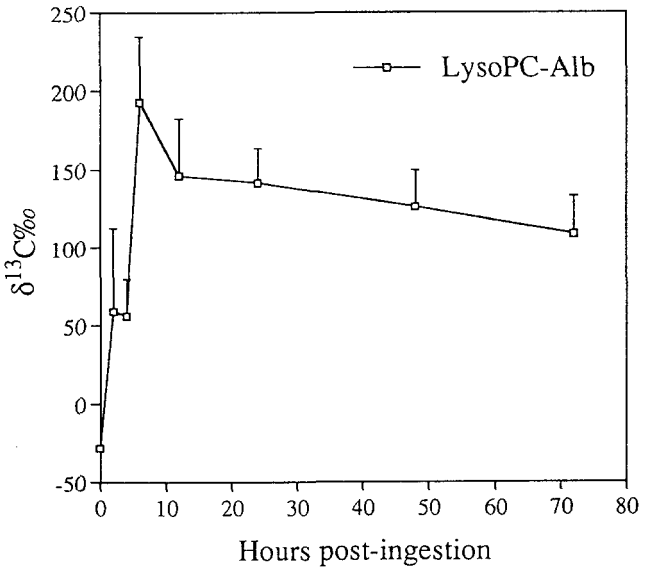

Fig. 2. Kinetics for the ^{13}C-22:6 abundance (δ^{13}C‰) in lyso-PC bound to albumin (Alb-lysoPC) in human subjects after ingestion of the ^{13}C-22:6-TG labeled at 7.72 atom %. Results are the mean ± SD of values from three human subjects.

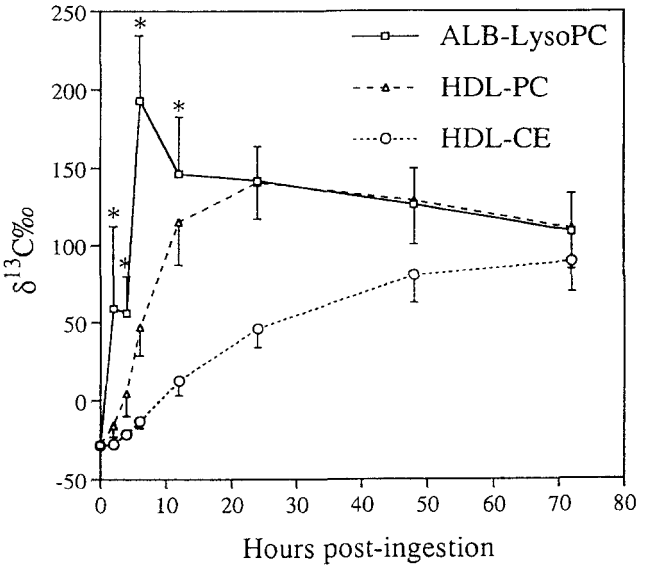

Fig. 4. Comparison of the kinetics for the ^{13}C-22:6 abundance (δ^{13}C‰) in lyso-PC bound to albumin (Alb-lysoPC), in HDL-phosphatidylcholine (HDL-PC), and in HDL-cholesteryl ester (HDL-CE). Results are the mean ± SD of values from three human subjects. *: the labeling in lyso-PC differed significantly from HDL-PC, $P < 0.005$.

cholesterol. As the PC fatty acid is mostly unsaturated at the *sn*-2 position and saturated at the *sn*-1 position, this reaction forms unsaturated CE and saturated lyso-PC. The The δ^{13}C‰ values were significantly higher in 22:6-lyso-PC than in 22:6-HDL-PC from 2 to 12 h. Therefore, the absence of a precursor–product relationship from HDL-PC to lyso-PC refuted the predominance of plasma LCAT activity in generating the [^{13}C]22:6-lyso-PC. At least, a part of these unsaturated lyso-PC must be synthesized from a highly enriched precursor.

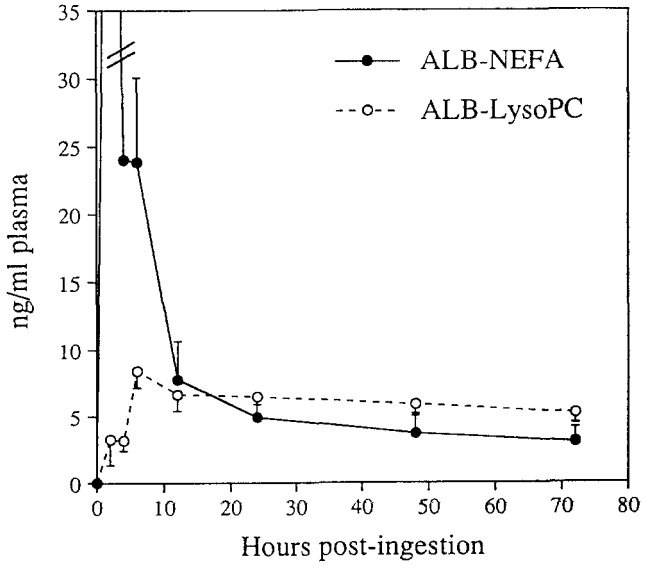

Fig. 3. Kinetics for the ^{13}C-22:6 concentration in nonesterified fatty acids bound to albumin (Alb-NEFA) and in lyso-PC bound to albumin (Alb-lysoPC). The ^{13}C-22:6 amount in each lipid class was calculated from the δ^{13}C‰ values and from the 22:6 endogenous concentrations. Each point represents the mean ± SD of three human subjects.

Uptake of [^{13}C]22:6 by blood cells

The labeling and accumulation kinetics of [^{13}C]22:6 were followed in platelet leukocyte and erythrocyte PL, after the ingestion of the tracer TG and were further compared to these kinetics in the two lipid classes bound to plasma albumin. The tracer ingestion induced a sharp rise in the labeling in platelet PC, occurring as soon as 1 h after intake, with a maximum at 12 h, followed roughly by a plateau until 72 h (**Fig. 5a**). A rapid increase of the δ^{13}C‰ values was also observed in platelet PE, but the labeling was significantly lower than in PC from 2 to 24 h. The highest labeling was observed in PI, in which these values were +42.95 ± 29.24, +75.20 ± 30.49, and +107.10 ± 22.10 δ^{13}C‰, at 2, 4, and 12 h, respectively (mean ± SD, n = 3). Calculated as the amount of [^{13}C]22:6 incorporated in each pool, it appears that [^{13}C]22:6 accumulated mostly in PE, the PL class where its endogenous location is the highest (Fig. 5b). Similar time courses of [^{13}C]22:6 appearance were observed in leukocyte PC and PE, with a significantly higher enrichment in PC than in PE (**Fig. 6**). In contrast, a slow increase of labeling occurred in erythrocyte PC after the tracer ingestion (**Fig. 7a**). The δ^{13}C‰ values increased from −27.66 ± 0.95 δ^{13}C ‰ at baseline to −6.36 ± 6.11 δ^{13}C‰ at 12 h, whereas they varied from −26.75 ± 0.08 to +89.43 ±

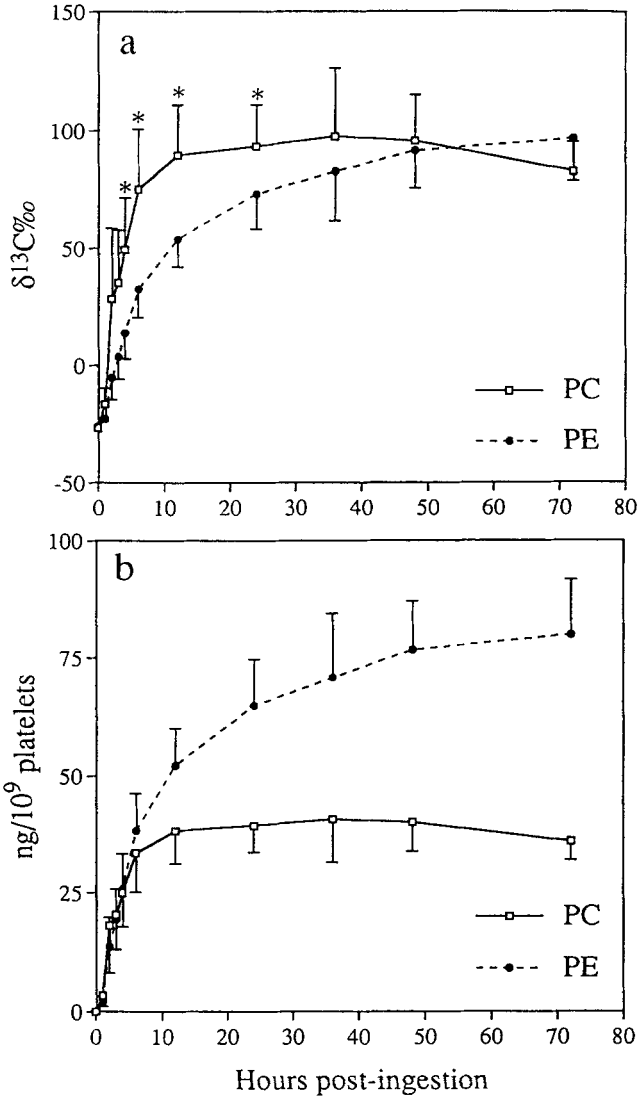

Fig. 5. Kinetics for the ^{13}C-22:6 abundance ($\delta^{13}C‰$) in platelet PC and PE. (a): the ^{13}C-22:6 abundance was measured by GCC-IRMS. *: the labeling in PC differed significantly from PE, $P < 0.005$. (b): The ^{13}C-22:6 concentration in PC and PE was calculated from the $\delta^{13}C‰$ values and from the endogenous 22:6 concentrations, quantitated by GC. Results are the mean ± SD of values from three human subjects.

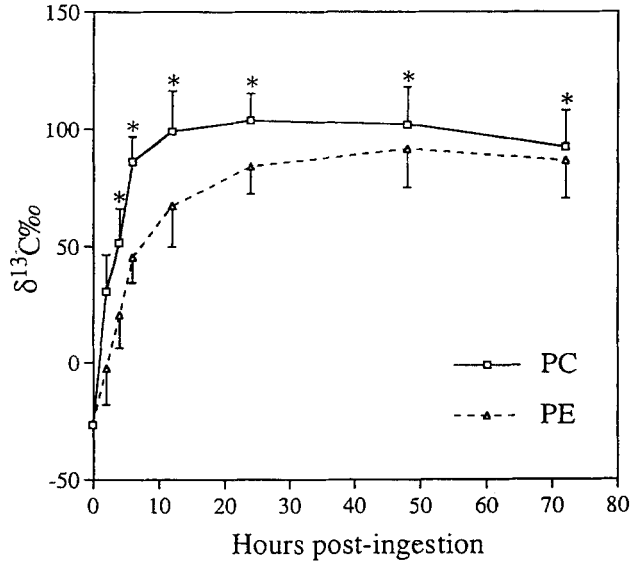

Fig. 6. Kinetics for the ^{13}C-22:6 abundance ($\delta^{13}C‰$) in leukocyte PC and PE. Results are the mean ± SD of values from three human subjects. *: the labeling in PC differed significantly from PE, $P < 0.005$.

21.43 $\delta^{13}C‰$ at 12 h in platelet PC. A rather slow ^{13}C-22:6 appearance occurred in erythrocyte PE, the labeling of which increased from −27.38 ± 0.42 $\delta^{13}C‰$, at baseline to −24.22 ± 1.11 $\delta^{3}C‰$, at 12 h. Consequently, in contrast to what was observed with platelets, the accumulation of [^{13}C]22:6 was weak in PE but occurred mostly in PC (Fig. 7b). Results in **Fig. 8** show the labeling kinetics of platelet and erythrocyte PC in comparison with the time course of the concentration of nonesterified [^{13}C]22:6 bound to albumin in plasma. [^{13}C]22:6 accumulated into platelet PC during the phase of maximal plasma circulation of [^{13}C]22:6 bound to albumin, while the labeling of erythrocyte PC was negligible over this time period. From 24 to 72 h, while the plasma concentration of [^{13}C]22:6 in NEFA dropped to its minimal concentration and the [^{13}C]22:6 in lyso-PC reached its highest level, the labeling plateaued in platelet PC, but still increased linearly without reaching a plateau at 72 h in erythrocyte PC. From the total amount of [^{13}C]22:6 accumulating in the two lipid species carried by albumin, it was computed that the relative contribution factor of NEFA involved in the [^{13}C]22:6 supply to platelets plus erythrocytes was γ_1 = 0.12 and that the relative contribution factor of lyso-PC to this supply was γ_2 = 1.0. The computed relative contribution factors of NEFA and lyso-PC to platelets were α = 0.32 and β = 0.0, respectively. The computed relative contribution factors of NEFA and lyso-PC to erythrocytes were $(1 - \alpha)$ = 0.68 and $(1 - \beta)$ = 1.0, respectively. It appears that the 22:6 supply to platelets occurs uniquely through the NEFA, whereas this pool is weakly involved in the 22:6 supply to erythrocytes as the product $(1 - \beta)\gamma_1$ was negligible compared to $(1 - \beta)\gamma_2$. In contrast, the lyso-PC pool is the primary pool involved with the 22:6 delivery to erythrocytes and represents the major part of this supply.

DISCUSSION

To investigate the steps involved in the plasma transport of 22:6 for cell incorporation in vivo in humans this PUFA was labeled with ^{13}C, a stable isotope of carbon, in order to establish precursor–product relation-

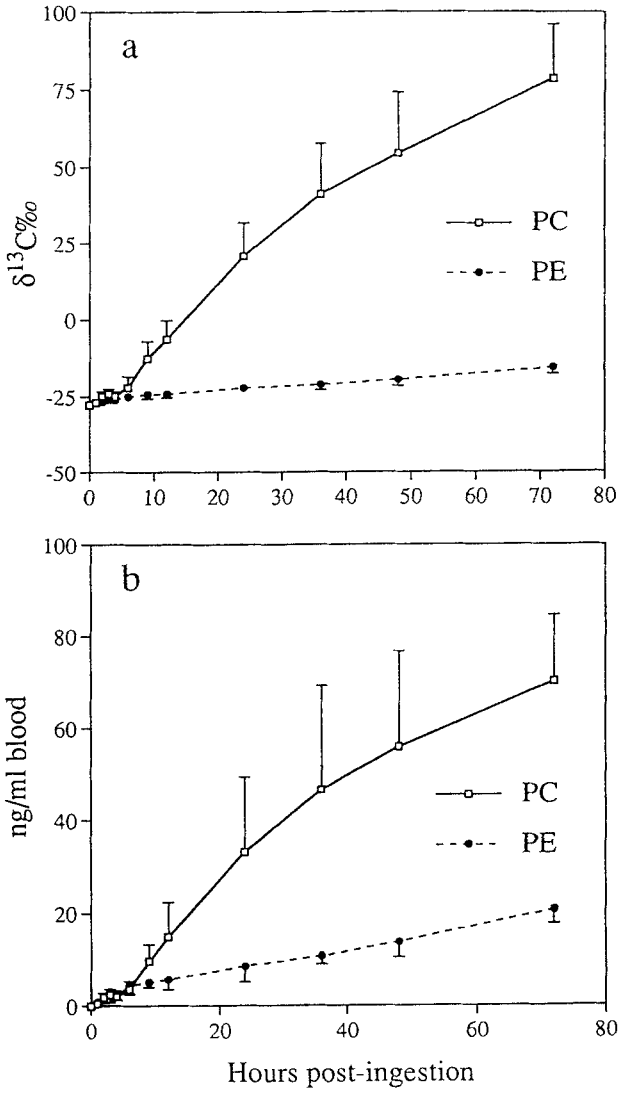

Fig. 7. Kinetics for the ^{13}C-22:6 abundance ($\delta^{13}C‰$) in erythrocyte PC and PE (a): The ^{13}C-22:6 was measured by GCC—IRMS. (b): The ^{13}C-22:6 concentration in PC and PE was calculated from the $\delta^{13}C‰$ values and from the endogenous 22:6 concentrations. Results are the mean ± of values from three human subjects.

ships within lipid classes. This approach presents the advantages of the non-toxic nature of stable isotopes and of high sensitivity of labeling detection which allows measurements of 0.001 atom % in lipid pools (13, 28).

The tracer fatty acid was given orally esterified in a TG which is the natural form of n–3 PUFA in fish oil. In view of the maximal [^{13}C]22:6 appearance at 2 h in the TG of a fraction containing both chylomicrons and VLDL, the [^{13}C]22:6 was rapidly absorbed by the enterocytes and the newly formed TG were transported to the blood by the lymphatic duct, according to the absorption pathway generally reported for these PUFAs (34). In studying the distribution of [^{13}C]22:6 within lipid classes after the tracer ingestion, we have previously shown that a low percentage of [^{13}C]22:6-TG from chylomicrons and VLDL was transferred to LDL during the processing of these lipoproteins and to HDL by cholesteryl ester transfer protein-mediated exchanges (N. Brossard, unpublished observation). Therefore, the sharp disappearance of these TG observed from 2 to 12 h was mostly due to their hydrolysis by the lipoprotein and hepatic lipases. Previous work from Griffiths et al. (35) has suggested that during chylomicron TG hydrolysis, meal-derived fatty acids are released in the plasma in addition to the tissue uptake. The release of lipoprotein lipase-derived fatty acids into the venous plasma in postabsorptive and postprandial states has also been described in humans (36). We report here that the [^{13}C]22:6 abundance was consistently and significantly lower in NEFA than in VLDL/chyl-TG, during the time course, which would be consistent with the predominant formation of nonesterified 22:6 bound to albumin from the VLDL/chyl-TG hydrolysis.

[^{13}C]22:6 in the NEFA pool, representing 7% of that present in VLDL/chyl-TG, is a potential substrate for incorporation in cells devoid of lipoprotein lipase. It could acylate the blood cell membrane lyso-PC, via the deacylation/reacylation pathway (19) and could represent the delivery route of 22:6 to the brain. The observation that albumin carried as much 22:6 in lyso-PC as in NEFA prompted us to follow the labeling of 22:6-lyso-PC and the level of [^{13}C]22:6-lyso-PC synthesized after the tracer intake. It appeared that the synthesis was rapid but with a maximum delayed from that of [^{13}C]22:6 in NEFA. Moreover, this lyso-PC accumulated while the nonesterified [^{13}C]22:6 reached its minimal concentration values, suggesting different synthesis pathways. Whereas it is well described that saturated lyso-PC in plasma are mostly formed as a result of the LCAT activity on the HDL-PC, the mechanism underlying the synthesis of unsaturated lyso-PC is unclear. Recent in vitro studies, using purified LCAT and defined PC molecular species, have shown that LCAT specificity for the hydrolysis of the *sn*-2 position of PC depends on the nature of the *sn*-2 acyl group, especially the chain length (37). For example, the human LCAT transfers the *sn*-1 fatty acid of 16:0–22:6-glycerophosphocholine to form 16:0-CE and 22:6-lyso-PC (37). In the present experiment, no attempt was made to distinguish between the [^{13}C]22:6 positions (*sn*-1 vs. *sn*-2) in the lyso-PC but it was clearly shown that the 22:6 labeling was significantly higher in lyso-PC than in HDL-PC, until 12 h after ingestion. Therefore, the high labeling of these 22:6-lyso-PC ensures the existence of another source of unsaturated lyso-PC than the plasma HDL-PC, likely a hepatic synthesis from a highly enriched precursor,

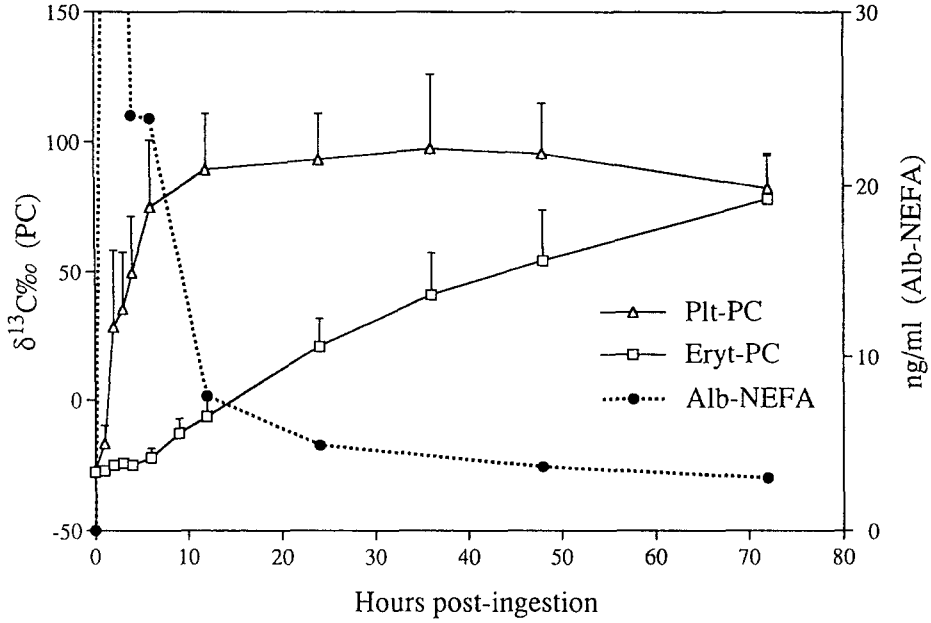

Fig. 8. The comparison of the kinetics for the ^{13}C-22:6 labeling in platelet-PC (Plt-PC) and erythrocyte PC (Eryt-PC) with the concentration of nonesterified ^{13}C-22:6 bound to albumin (Alb-NEFA) in plasma, after ingestion of ^{13}C-22:6-TG. Results are the mean ± SD of values from three human subjects.

which agrees with the observation that albumin stimulates the release of unsaturated lyso-PC from isolated perfused liver and cultured hepatocytes (38, 39). Unsaturated lyso-PC species would be formed in the liver either by the activity of the hepatic triglyceride lipase (40) or by that of an intracellular phospholipase A_1 (39) or by a combination of both activities. A physiological function of this release of unsaturated lyso-PC could be the supply of extrahepatic tissues with choline and/or unsaturated fatty acids. As [^{13}C]22:6-lyso-PC was efficiently formed after the tracer ingestion, lyso-PC could be another carrier of 22:6 to tissues, especially the brain, accounting for the preferential location of 22:6 in this organ.

The nonesterified [^{13}C]22:6 and [^{13}C]22:6-lyso-PC bound to albumin were specifically involved in the [^{13}C]22:6 uptake by platelets/leukocytes and erythrocytes, respectively. The appearance of [^{13}C]22:6 in erythrocyte PL is of interest as it has been hypothesized that it may reflect the 22:6 brain uptake (12, 22). The rapid increase of 22:6 labeling in platelet PC was mostly due to the [^{13}C]22:6 in NEFA bound to albumin. Although an efficient cycle of deacylation/reacylation has been extensively described in platelets in vitro and used to modify the fatty acid composition of these cells (19), the occurrence of this Lands pathway in vivo in human has been questionned (41, 42). Von Schacky and Weber (41) have shown that feeding 6 g/day of pure eicosapentaenoic acid or 22:6 to healthy volunteers induced an increase of these PUFA in plasma PL and NEFA, 4 h after ingestion, whereas they did not incorporate in platelet PC and PE until day 6. These authors suggested that platelets do not take up n–3 PUFA from the surrounding plasma but rather their fatty acid composition may be determined during the megacaryocyte maturation. From our results it is evident that platelets took up 22:6 from plasma NEFA, through the deacylation/reacylation cycle. Plasma NEFA are activated by acyl-CoA synthetases into acyl-CoA and are then substrates of acyl-CoA transferases to reacylate membrane lyso-PC. Platelets contain a high affinity acyl-CoA synthetase, specific for eicosanoid precursor fatty acids (43) and an acyl-CoA transferase that is also selective for the unsaturated acyl-CoA (44). [^{13}C]22:6 was activated and esterified in vivo, despite the high concentration of other fatty acids in NEFA bound to albumin. How these enzymes are regulated and why a mass increase of 22:6 was not detected in human platelets in vivo by including it in the diet for a short period of time (41) remains to be determined. A high and rapid [^{13}C]22:6 enrichment in PE was also detected immediately after ingestion. The [^{13}C]22:6 abundance in PC, which was significantly higher than in PE, suggests a relationship between [^{13}C]22:6-PC and [^{13}C]22:6-PE, and the PE remodeling through the transacylase activities between these two phospholipids in vivo. Moreover, the deacylation/reacylation of PE has not been reported in vitro, presumably due to the low PLA_2 activity on this PL, and it

is likely that the [^{13}C]22:6 accumulated in PE through its transfer from PC to PE (19, 45). Whereas this transfer was low in isolated platelets incubated with [^{14}C]22:6 (31), it was very active in vivo, as half of the [^{13}C]22:6 incorporated in PC was transferred into PE from 2 to 6 h, to finally accumulate primarily in PE. PI presented the highest 22:6 labeling, although feeding experiments reported no accumulation of 22:6 and eicosapentaenoic acid after fish oil supplementation, in humans (41, 46). These PUFAs were also highly labeled in PI from radiolabeled platelets PUFA (47, 48). The discrepancy between these results may be explained by a very high 22:6 turn-over in PI. The [^{13}C]22:6 appearance in platelet and leukocyte PC and PE being quite comparable, these two cell types probably share the same PL remodeling pathways, at least for 22:6-PL.

In contrast to the case in platelets and leukocytes, the relative contribution of NEFA for the [^{13}C]22:6 supply to erythrocytes was low. Possible mechanisms for PL renewal in erythrocytes include the PL deacylation/reacylation (49), and the uptake of lyso-PC followed by reacylation in the membrane (50). Although the relative importance of these pathways is not clear in vivo, the uptake of entire PL molecules is predominant in vitro, with the exchange of lyso-PC being far more active than the other PL classes and PE not being transferred (50). Based on these observations and on the relative contribution factor values of NEFA and lyso-PC to the [^{13}C]22:6 supply to erythrocytes, we suggest that lyso-PC might efficiently deliver 22:6 to erythrocytes during their circulating life span. The erythrocyte fatty acid composition is generally taken as a tentative index of brain fatty acid modification (22). We have previously reported the kinetics of [^{13}C]22:6-lyso-PC appearance in rat plasma after the ingestion of [^{13}C]22:6-TG (13). This labeling kinetics was compared to that of 22:6 in NEFA bound to albumin, in the brain and erythrocyte PC. When [^{13}C]22:6-lyso-PC reached its highest concentration in plasma, the [^{13}C]22:6 incorporation in brain PC increased linearly although the nonesterified [^{13}C]22:6 bound to albumin had already dropped to its lowest plasma level. Moreover, in the young rat, the injection of [^{3}H]22:6-lyso-PC bound to albumin induced a 10-fold higher uptake of [^{3}H]22:6 to the brain than the injection of [^{3}H]22:6 bound to albumin (51). Therefore, as the kinetics of [^{13}C]22:6 appearance in NEFA, lyso-PC, and erythrocyte PC were quite comparable in humans and rats, we suggest that lyso-PC may be another carrier of [^{13}C]22:6 to the brain in humans.

On the other hand, the [^{13}C]22:6 accumulation was low in erythrocyte PE, in contrast to platelets and leukocytes. The reasons for this might be that the transacylases are virtually absent in erythrocytes or differently regulated, but it could also involve the pathways by which the fatty acid is first introduced into the membrane and consequently its inner/outer leaflet location. Erythrocyte membranes consist of one external plasma membrane that incorporates PC in the outer leaflet by exchanges with plasma. Van den Boom et al. (49) have suggested that the renewal of unsaturated PC in rabbit erythrocytes would be almost exclusively due to exchanges with the plasma and confined to those species (around 80%) present in the outer leaflet, the transfer of the incorporated PC to the inner leaflet being very slow. In contrast, we have shown that the renewal of [^{13}C]22:6-PC in platelets occurs mostly by the deacylation/reacylation pathway. This process occurs at the inner leaflet containing about 50% of the PC molecules that would be accessible for the transacylation reaction involving PE which is primarily located on the cytoplasmic side of the membrane.

The data presented here show that in humans, the NEFA bound to albumin are the main source of 22:6 for platelets and leukocytes during their circulating life span, whereas we hypothesize that the brain would take up a fraction of its 22:6 from albumin lyso-PC as it is the case for erythrocytes.

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