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L-Carnitine Dissimilation in the Gastrointestinal Tract of the Rat†

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ABSTRACT: Results of previous studies in this laboratory and others have suggested that L-carnitine is degraded in the gastrointestinal tract of the rat, perhaps by the action of indigenous flora. L-[*methyl*-^{14}C]Carnitine was administered to rats either orally or intravenously in doses of 86 nmol or 124 μmol, and expired air, 48-h urine and fecal collections, and selected tissues at 48 h after isotope administration were examined for radiolabeled carnitine and metabolites. Urine and feces of rats receiving oral L-[*methyl*-^{14}C]carnitine consistently contained two radiolabeled metabolites which were identified as trimethylamine *N*-oxide (primarily in urine) and γ-butyrobetaine (primarily in feces). In these rats, these metabolites accounted for up to 23% and 31% of the administered dose, respectively. By contrast, for rats receiving intravenous L-[*methyl*-^{14}C]carnitine or germ-free rats receiving the isotope orally or intravenously, virtually all of the radioactivity recovered was in the form of carnitine. Analyses for $^{14}CO_2$ and [^{14}C]trimethylamine in expired air revealed little or no (less than 0.1% of dose) conversion to these compounds, regardless of size of dose or route of administration. Results of this study demonstrate conclusively that L-carnitine is degraded in the gastrointestinal tract of the rat and that indigenous flora are responsible for these transformations.

L-Carnitine [β-hydroxy-γ-(*N*-trimethylammonio)butyrate] is a natural constituent of higher organisms and, in particular, cells of animal origin. Its primary function is to transfer long-chain fatty acids, as acylcarnitine esters, across the mitochondrial inner membrane. L-Carnitine is found in many foodstuffs and is particularly abundant in meat and dairy products. This compound is synthesized endogenously from lysine and methionine.

In mammals, carnitine is excreted primarily in urine. Greater than 95% of total carnitine excreted in urine and feces of dogs or humans was present in urine (Rebouche & Engel, 1983, 1984). Carnitine metabolism in rats (Cederblad &

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0006-2960/84/0423-6422$01.50/0

Lindstedt, 1976; Brooks & McIntosh, 1975), dogs (Rebouche & Engel, 1983), and humans (Rebouche & Engel, 1984) has been studied by the technique of kinetic compartmental analysis. The rates of carnitine elimination determined by kinetic analysis were higher than the rates of excretion determined by chemical analysis of urine and feces of these species. This technique did not discriminate between catabolic and excretory routes for L-carnitine disposal. Thus, overestimation of carnitine excretion by kinetic analysis may reflect activity of catabolic pathways for carnitine. Support for this hypothesis rests in the observation that for 8 of 11 human subjects excretion of carnitine in urine and feces was lower than dietary carnitine intake (Rebouche & Engel, 1984). However, intravenously-administered radiolabeled carnitine was not significantly degraded in dogs (Yue & Fritz, 1962; Rebouche & Engel, 1983), rats (Brooks & McIntosh, 1975; Cederblad & Lindstedt, 1976), or humans (Rebouche & Engel, 1984). These results and the knowledge that certain microorganisms, including some species normally found in the mammalian intestine, are able to degrade L-carnitine led us to speculate that dietary carnitine is partially degraded in the gastrointestinal tract. This hypothesis is consistent with the virtual absence of radiolabeled carnitine degradation products following intravenous administration of [^{3}H]- or [^{14}C]carnitine, as systemic carnitine would be affected only to the extent that it enters the intestinal lumen. This hypothesis may at least in part explain the discrepancy in mammals between carnitine excretion determined chemically and carnitine elimination calculated by kinetic analysis.

Materials and Methods

Materials. L-[*methyl*-^{14}C]Carnitine was obtained from Amersham Corp. (Arlington Heights, IL). [^{14}C]Toluene internal standard was purchased from New England Nuclear Corp. (Boston, MA). Trimethylamine *N*-oxide was obtained from Sigma Chemical Co. (St. Louis, MO), and γ-butyrobetaine [(3-carboxypropyl)trimethylammonium chloride] was provided by Aldrich Chemical Co. (Milwaukee, WI). L-Carnitine was a generous gift from Dr. Claudio Cavazza, Sigma Tau, Rome, Italy.

Conventional male rats (Sprague-Dawley strain) were obtained from Harlan-Sprague-Dawley, Inc. (Indianapolis, IN). Germ-free, male rats (Fischer strain) were purchased from Charles River Breeding Laboratories, Willmington, MA.

Treatment of Animals. Five groups of rats (two to six rats per group) weighing 95–191 g [147 ± 14 g (mean ± SD) for conventional rats; 95 and 101 g for germ-free rats] were given oral or intravenous doses of L-[*methyl*-^{14}C]carnitine (5 μCi). A "tracer dose" consisted of 86 nmol of carnitine (specific activity 58 Ci/mol), and a "bolus dose" provided 124 μmol (0.04 Ci/mol). Intravenous doses were administered under light ether anesthesia via the tail vein in a total volume of 0.25–0.30 mL of water. Oral doses were prepared in 50–100 μL of water and were administered through a disposable pipet tip.

Rats were housed in glass metabolism cages (for collection of expired air) or polycarbonate metabolism cages for other experiments. Urine and feces were collected separately for 48 h following administration of the dose. Food (stock laboratory rat diet) and water were offered ad libitum. At 48 h after administration of the dose, the animals were sacrificed, and tissues (liver, kidney, heart, and a portion of skeletal muscle) were excised, weighed, cut into 100–300-mg pieces, and frozen in liquid nitrogen. The entire intestinal tract, including the caecum and contents, was removed, weighed, and frozen in liquid nitrogen. These specimens and the urine and fecal collections were stored at −80 °C for analysis.

Analysis of Tissue, Urine, and Fecal Specimens. Tissue aliquots (0.3–1.1 g, except intestine) were extracted with 20 volumes (w/v) of chloroform/methanol (3:2) (Tanphaichitr et al., 1971) by homogenization using a Tissumizer (Tekmar Co., Cincinnati, OH) for 2 × 30 s at half-maximum speed. The homogenates were filtered through Whatman 1 filter paper. The blender cup, filter, and residue were washed with 2 × 10 volumes (initial tissue weight, w/v) of chloroform/methanol (3:2). The filtrates were combined and evaporated in vacuo. The residues were taken up in 10 mL of 0.1 N KOH in 50% aqueous ethanol and were heated for 1 h at 56 °C to hydrolyze carnitine esters. The mixtures were neutralized with 1 N HCl and were evaporated to near dryness. To the tissue extracts was added 2.5 mL of 0.25 N (in sodium ions) sodium citrate, pH 4.08, containing 1% poly(ethylene glycol 400). The mixtures were filtered through glass wool to remove coagulated lipid, and the filtrate volumes were adjusted to 5 mL with water.

Whole intestine and fecal collections were homogenized (4 × 1 min) in 10 volumes (w/v) of chloroform/methanol (3:2) by using a Waring blender with a stainless-steel cup. The intestine extracts were prepared as above, except that the aqueous extracts (~20 mL) after hydrolysis were clarified by extraction with ether (approximately 20 mL, 3 times) and centrifugation (105000*g*, 15 min). The final volume of the extracts was 25 mL and contained 12.5 mL of citrate buffer (as above). The residue following filtration of the fecal homogenate was reextracted with 200 mL of water, and the mixture was centrifuged at 1860*g* for 10 min. Half of the aqueous extract and half of the organic extract were combined, evaporated to dryness in vacuo, and prepared for chromatography as described above for intestine.

Aliquots of urine were made 0.1 N in KOH by addition in 1 N KOH and were heated at 56 °C for 1 h. The hydrolysate was neutralized with 1 N HCl, and citrate buffer (2.5 mL) was added, as above. The volume was adjusted to 5 mL by evaporation in vacuo or by addition of water.

Quantitation of [^{14}*C*]*Carnitine and Metabolites.* For quantitation of [^{14}C]carnitine and nonvolatile, radioactive metabolites, tissue, urine, and fecal extracts (1–8 mL) were chromatographed on a cation-exchange resin column as previously described (Rebouche & Engel, 1980).

For analysis of radioactivity in expired air, a house vacuum line was placed to draw gases from the metabolism chamber into a series of four gas washing bottles containing 100 mL each of 1 N NaOH (first two bottles) or 1 N HCl (second set of two bottles). A humidified air inlet was provided at the top of the chamber. Each solution was changed at 2, 6, and 12 h after administration of the dose. Half-milliliter aliquots of NaOH solutions (for collection of $^{14}CO_2$) were counted as a thixotropic gel (Harlan, 1963), and 1-mL aliquots of HCl solutions (for trapping of [^{14}C]trimethylamine) were counted in 10 mL of 3a70 (Research Products International, Downers Grove, IL). [^{14}C]Toluene was used as an internal standard.

Identification of ^{14}*C-Labeled Metabolites of* L-[*methyl*-^{14}*C*]*Carnitine.* Urine and fecal metabolites were purified for analysis by cation-exchange column chromatography and preparative thin-layer chromatography.

Mass spectra of the urine and fecal metabolites were obtained with a Kratos MS 50/DS 55 mass spectrometer/computer system operating at 6-kV accelerating potential. Spectra for the urine metabolite and standard compound were generated by electron ionization at 70 eV. The sample was placed directly on the insertion probe, and accurate mass data were

Table I: Carnitine Metabolism in the Rat[a]

dose: route:	tracer oral	bolus oral	tracer intravenous	bolus intravenous	bolus[d] oral
		Total[b]			
radioact. recovered[e]	69.1 ± 8.2 (3)	76.8 ± 10.2 (3)	71.6 ± 3.6 (3)	83.0 ± 13.3 (3)	91.1 (2)
[^{14}C]carnitine[f]	81	65	96	97	99
		Tissues[c]			
radioact. recovered[e]	52.9 ± 9.3 (6)	14.5 ± 4.4 (5)	66.4 ± 3.6 (3)	15.1 ± 1.3 (3)	16.4 (2)
[^{14}C]carnitine[f]	98	88	99	94	100
		Urine			
radioact. recovered[e]	9.73 ± 9.03 (6)	28.8 ± 10.7 (6)	3.86 ± 1.56 (3)	67.4 ± 12.0 (3)	17.7 (2)
[^{14}C]carnitine[f]	39	34	76	98	99
		Feces			
radioact. recovered[e]	4.28 ± 2.40 (3)	53.2 ± 6.7 (3)	1.45 ± 0.74 (3)	0.526 ± 0.240 (3)	56.8 (2)
[^{14}C]carnitine[f]	16	55	21	34	99

[a] All values are mean ± SD, with number of rats studied in parentheses. [b] Sum of total radioactivity in skeletal muscle, heart, liver, kidney, and intestine plus luminal contents, 48-h urine and feces collections. [c] Sum of skeletal muscle, heart, liver, kidney, and intestine plus luminal contents. [d] Germ-free rats. [e] Percent of dose. [f] Percent of radioactivity recovered.

collected by the computer system. Spectra for the fecal metabolite and standard compound were obtained by fast atom bombardment mass spectroscopy. The sample was prepared in a glycerol matrix and was deposited on a copper-tipped direct insertion probe. A primary atom beam of Xe was produced by using a saddle field ion source (TAB11NF, Ion Tech, Ltd., Teddington, U.K.) operating with a tube current of ~1.5 mA at an energy of ~7 keV.

Results

Recovery of Radioactivity. To determine the efficiency of recovery of radioactivity from tissues and feces in the chloroform/methanol extraction procedure, residues after filtration were reextracted with water, and aliquots of the aqueous extracts were counted. The organic extracts of tissue contained 71–96% of total radioactivity (mean ± SD, 83 ± 8%; N = 25). For skeletal muscle, which accounted for 65–91% (86 ± 6%, mean ± SD; N = 19) of total radioactivity in tissues, 82 ± 3% (mean ± SD, N = 5) was extracted into chloroform/methanol. Extraction of radioactivity in feces by chloroform/methanol was variable and considerably less efficient than for extraction of tissues. Indeed, up to 64% of total fecal radioactivity was recovered in aqueous extracts of fecal residues following the organic extraction procedure. Therefore, we prepared organic extracts of fecal collections and aqueous extracts of the residues after organic extraction and combined aliquots of the two to prepare a final extract for analysis.

In individual rats, radioactivity recovered in tissues, feces, and urine ranged from 61% to 97% of the administered dose (mean values for groups of rats are tabulated in Table I). Bolus doses of carnitine tended to be excreted rapidly in feces (oral administration) or urine (intravenous or oral administration). Large percentages of tracer does of carnitine were retained in tissues, irrespective of the route of administration. Analyses for $^{14}CO_2$ and [^{14}C]trimethylamine in expired air revealed little or no (less than 0.1% of the dose) conversion of L-[*methyl*-^{14}C]carnitine to these compounds, regardless of the size of the dose or the route of administration.

Quantitation of [^{14}C]Carnitine and Metabolites. Tissue, urine, and fecal extracts were analyzed by ion-exchange column chromatography and liquid scintillation counting. Virtually all of the radioactivity in tissues was in the form of [^{14}C]carnitine (Table I), regardless of the size of the dose or the route of administration. In urine of rats receiving a bolus dose of [^{14}C]carnitine orally, only 34% of the radioactivity recovered was [^{14}C]carnitine, compared to 98% in rats receiving a bolus dose intravenously and 99% in germ-free rats given an oral bolus dose. Rats receiving a bolus dose of [^{14}C]carnitine orally excreted 53% of the radioactivity in feces, and only 55% of that excreted was in the form of [^{14}C]-carnitine. In these rats, the amount of carnitine passing through the intestinal lumen apparently exceeded the capacity of the tissue for absorption. In germ-free rats given the same dose orally, 57% was excreted in feces, but greater than 99% was in the form of [^{14}C]carnitine. By contrast, conventional rats given intravenous doses or a tracer dose orally of [^{14}C]-carnitine excreted less than 5% of the dose in feces. However, in each group greater than 65% of the radioactivity in feces was in a chemical form other than [^{14}C]carnitine.

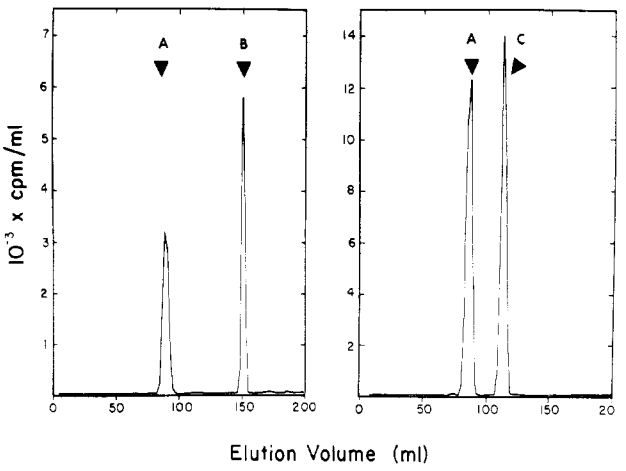

FIGURE 1: Chromatographic analysis of urinary and fecal excretion of L-[*methyl*-^{14}C]carnitine and metabolites. Chromatographic conditions were a 0.9 × 50 cm column of AG50WX8 resin (Bio-Rad, 200–400 mesh), Na^+ form, equilibrated with 0.25 M (in sodium ions) sodium citrate, pH 4.08, and temperature equilibrated at 50 °C. The column was eluted with a linear gradient of 125 mL of 0.25 M (in sodium ions) sodium citrate (pH 4.08) with 1% poly(ethylene glycol) 400) and 125 mL of 0.25 N NaOH. Two-milliliter fractions were collected. Chromatograms shown are from a rat given a bolus dose of 5 μCi of L-[*methyl*-^{14}C]carnitine orally. Left panel, urine extract; right panel, fecal extract.

In urine chromatograms, two peaks of radioactivity were consistently observed (Figure 1, left panel). The first peak (A) corresponded to carnitine and the second (B) to a metabolite. In chromatograms of fecal extracts, two peaks also were observed (Figure 1, right panel). The first peak (A) corresponded to carnitine and the second (C) to a metabolite, but the metabolite in feces was different from that found in urine. Quantitatively, the urine and fecal metabolites of [^{14}C]carnitine in individual rats accounted for up to 23% and

Table II: Quantitation of L-Carnitine Metabolites[a]

dose	route	% of dose converted to: trimethylamine N-oxide (urine)	% of dose converted to: γ-butyrobetaine (feces)
bolus	oral	11.4 ± 7.4 (6)	23.1 ± 8.1 (3)
tracer	oral	3.59 ± 5.48 (6)	2.90 ± 1.88 (3)
bolus	intravenous	0.627 ± 0.352 (3)	0.238 ± 0.110 (3)
tracer	intravenous	0.579 ± 0.341 (3)	0.910 ● 0.523 (3)
bolus[b]	oral	0.095 (2)	0 (2)

[a] All values are mean ± SD, with the number of rats studied in parentheses. [b] Germ-free rats.

Table III: Comparison of Chromatographic Properties of Radioactive Metabolites of L-[*methyl*-^{14}C]Carnitine and Reference Compounds

compound	R_f, thin-layer chromatography A[a]	B[b]	C[c]	R_f, paper chromatography[d]	elution volume, liquid column chromatography[e]
metabolite from urine	67	62	61	57	150
trimethylamine *N*-oxide	66	63	62	56	
metabolite from feces	44	49	46	50	120
γ-butyrobetaine	42	46	47	47	120
L-carnitine	36	57	32	42	90

[a] Solvent system: methanol/concentrated NH_4OH (75:25). Medium: silica gel G (Analtech, Inc., Newark, DE), 5 × 20 cm glass plates, developed to 15 cm; visualized with I_2 vapor. [b] Solvent system: methanol/acetone/concentrated HCl (90:10:4). [c] Solvent system: phenol (liquified)/1-butanol/concentrated NH_4OH, (50:50:20). [d] Solvent system: 1-butanol/glacial acetic acid/water (60:15:25). Medium: Whatman 3MM paper, descending, developed to 25 cm; visualized with I_2 vapor. [e] Chromatographic conditions are described in the legend to Figure 1.

31%, respectively, of the administered dose (mean values for groups of rats are reported in Table II).

Identification of Urine and Fecal Metabolites. Preliminary identification of isolated radioactive metabolites from urine and feces was obtained by thin-layer, paper, and ion-exchange column chromatographic comparisons of the metabolites with reference compounds (Table III). The metabolite from urine was found to behave identically with trimethylamine *N*-oxide in four different chromatographic systems, and the metabolite from feces was indistinguishable from γ-butyrobetaine in five systems.

Electron ionization mass spectra of the metabolite from urine and authentic trimethylamine *N*-oxide gave molecular ions ($M^{+\cdot}$) at $m/z = 75$ for each sample. Also for each sample, $(M - OH)^+$ and $(M - CH_3O)^+$ were observed at $m/z = 58$ and 42, respectively. Fast atom bombardment mass spectra of the metabolite from feces and authentic γ-butyrobetaine gave positive ions at $m/z = 146$. On the basis of these data, the structures of the metabolites from urine and feces were assigned as trimethylamine *N*-oxide and γ-butyrobetaine, respectively.

Discussion

L-Carnitine is degraded by a variety of microorganisms. End products of microbial carnitine dissimilation and organisms shown to effect these transformations include trimethylamine in *Serratia marcescens* (Unemoto et al., 1966), *Acinetobacter calcoaceticus*, and *Pseudomonas putida* (Miura-Fraboni et al., 1982), glycine betaine in *Pseudomonas putida* (Miura-Fraboni, 1982), γ-butyrobetaine in *Escherichia coli*, *Citrobacter freundii*, several *Salmonella* species, *Proteus vulgaris*, and *Proteus mirabilis* (Seim et al., 1980), and carbon dioxide and water in *Acinetobacter calcoaceticus* and *Pseudomonas putida* (Miura-Fraboni et al., 1982).

Experiments in which mice and rats were given heavy oral loads of L-carnitine have shown increased urinary excretion of trimethylamine, trimethylamine *N*-oxide, and γ-butyrobetaine (Strack et al., 1963; Strack & Seim, 1979). However, a direct precursor–product relationship was not established. Bremer (1983) suggested that these metabolites were formed by intestinal microorganisms. Indeed, it is well-known that choline is degraded to trimethylamine by mammalian intestinal flora (Marks et al., 1977).

In the present study, we have shown unequivocally that γ-butyrobetaine and trimethylamine *N*-oxide are formed from L-carnitine. We conclude that endogenous intestinal flora are responsible for these transformations, on the basis of the absence of degradation of carnitine in germ-free rats and on the greatly decreased level of dissimilation of L-[*methyl*-^{14}C]-carnitine when administered intravenously compared to orally in conventional rats. Further evidence for this conclusion is found in the substantial degradation of the relatively small amount of [^{14}C]carnitine which enters the intestinal tract and is excreted in feces of rats receiving an intravenous dose of this compounds. By analogy with choline degradation in mammals (Marks et al., 1977), trimethylamine formed from carnitine in the gastrointestinal tract may be absorbed and taken up by the liver where it is oxidized by a microsomal amine oxidase (Baker & Chaykin, 1962).

The extent of metabolism of orally administered carnitine was related to the size of the dose. This result was probably due to less efficient absorption of carnitine at the higher level of administration.

The results of this study at least partially explain the anomalous differences between carnitine intake and excretion in rats, dogs, and humans. Furthermore, the results have implications for individuals taking chronically high doses of carnitine orally (either as a "health-food" supplement or for treatment of genetic or acquired carnitine deficiency). In these subjects, microbial enzymes for carnitine dissimilation may be induced, leading to a more dramatic rate of carnitine degradation than observed in this study in rats. This mechanism may explain the "fishy" odor (probably resulting from trimethylamine production and expiration) which is sometimes associated with these individuals.

Acknowledgments

Mass spectral analyses and interpretations were provided by Dr. Ian Jardine and Gale Scanlon of the Mayo Foundation Grainger Mass Spectrometry Facility.

Registry No. L-Carnitine, 541-15-1; trimethylamine *N*-oxide, 1184-78-7; γ-butyrobetaine, 407-64-7.

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Pyrenedodecanoylcarnitine and Pyrenedodecanoyl Coenzyme A: Kinetics and Thermodynamics of Their Intermembrane Transfer†

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ABSTRACT: The intermembrane transfer kinetics and transition-state thermodynamics of pyrenedodecanoylcarnitine (PDC), pyrenedodecanoyl coenzyme A (PDCoA), and pyrenedodecanoic acid (PDA) were measured by observing the time-dependent decay in pyrene excimer fluorescence. Probe molecules transferred more slowly with an increase in vesicle size. Rates of PDC and PDA transfer were increased from a liquid lipid phase when compared to a gel phase, while a saturated lipid phase had variable effects on the transfer kinetics when compared to an unsaturated lipid vesicle. Increasing vesicle surface charge by the introduction of phosphatidylserine (PS) into the vesicle matrix had two distinct effects: (i) a decrease in PDC transfer rates as the PS concentration increased and (ii) an initial increase in transfer rates of the amphiphilic anions PDA and PDCoA, followed by a decrease as the PS content increased. Transfer from natural membranes (cardiac and hepatic reticular and mitochondrial membranes) was markedly decreased (up to 35-fold) when compared to large phospholipid vesicles. These decreases in rates were accompanied by significant increases in the transition-state free energies. Finally, the pyrenedodecanoate esters had critical micelle concentrations similar to the natural long-chain esters, i.e., palmitate. In the presence of acceptor vesicles all probes showed only slight accessibility to quenching by the aqueous quencher nicotinamide.

The relationship between the biochemical mechanisms controlling the oxidation/esterification processes and the physical interaction of lipid metabolites with their natural matrix, i.e., cellular membranes, is important to the study of lipid metabolism. The amphipathic properties of active lipid metabolites including fatty acids, fatty acyl-CoA, and fatty acylcarnitine and the membrane-bound nature of the enzymes of their metabolism necessitate their interaction in specific cell membrane environments. The location and concentration of these molecules in cell membranes must be related to the metabolic state of the cell, amphiphile availability, and the probability of metabolite–membrane interaction. Therefore, a thorough description of fatty acid metabolism requires a delineation of the physical processes controlling the kinetics of movement and thermodynamics of association of fatty acid metabolites between the various intracellular membrane compartments.

Pyrene esters of fatty acids represent one group of probe molecules that allows direct observation of amphiphile movement via concentration-dependent excimer fluorescence (Doody et al., 1980; Roseman & Thompson, 1980). With this method the rates of intervesicular transfer of pyrenedodecanoic acid and its coenzyme A and carnitine derivatives can be measured. The thermodynamics of the activated state are derived from absolute rate theory. Differences in transfer kinetics can then be correlated with differences in the structure of the transferring amphiphile and the microenvironment from which it is transferred.

Materials and Methods

Materials. Pyrenedodecanoic acid was purchased from Molecular Probes Inc., Junction City, OR. Dicyclohexylcarbodiimide and 4-pyrrolidinopyridine were obtained from Aldrich Chemical Co., Milwaukee, WI. All phospholipids were obtained from Avanti Polar Lipids Inc., Birmingham, AL. Carnitine was obtained from Tridom-Fluka Inc., Hauppaugge, NY. Coenzyme A and nicotinamide were purchased from Sigma Chemical Co., St. Louis, MO, and Sephadex G-50 was obtained from Pharmacia Fine Chemicals Inc., Piscataway, NJ. All other reagents were of standard laboratory grade.

Synthesis of Probes. Pyrenedodecanoyl coenzyme A (PDCoA) and pyrenedodecanoylcarnitine (PDC) were synthesized from pyrenedodecanoic acid (PDA) by using the procedure of Patel et al. (1979) as previously described (Wolkowicz et al., 1982). Briefly, the fatty acyl anhydride was prepared in distilled tetrahydrofuran by mixing PDA (0.3 mmol) and dicyclohexylcarbodiimide (0.15 mmol). The dicyclohexylurea precipitate was removed by filtration and the filtrate added to coenzyme A or carnitine suspended in warm

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0006-2960/84/0423-6426$01.50/0