(1-Pyrenebutyryl)carnitine and 1-Pyrenebutyryl Coenzyme A: Fluorescent Probes for Lipid Metabolite Studies in Artificial and Natural Membranes†

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ABSTRACT: Membrane properties of fatty acyl coenzyme A (CoA) and acylcarnitine have been studied with 1-pyrenebutyryl-CoA and (1-pyrenebutyryl)carnitine. These molecules have the spectroscopic properties of pyrene and its derivatives and exhibit biological and chemical characteristics related to the acyl esters. (1-Pyrenebutyryl)carnitine (PBC) is more soluble in nonpolar solvents than 1-pyrenebutyryl-CoA (PB-CoA), and critical micelle concentrations of both compounds resemble the medium-chain fatty acyl esters. PB-CoA inhibits phosphorylating (ADP-stimulated) respiration in rat liver mitochondria noncompetitively (K_I = 2 μM) and carnitine palmitoyl-CoA and octanoyl-CoA transferases competitively (K_I = 2.1 μM and 15 μM, respectively). PBC does not inhibit carnitine palmitoyl-CoA transferase or mitochondrial respiration when glutamate–malate or succinate (+rotenone) is used as the respiratory substrate. PBC is a potent inhibitor of phosphorylating respiration with either palmitoylcarnitine (I_{50} = 1.4 μM) or octanoylcarnitine (I_{50} = 40 nM) as the respiratory substrate. The mitochondrial carnitine–acylcarnitine translocase is competitively inhibited by PBC with a K_I = 0.6 μM for palmitoylcarnitine exchange and 23 nM for carnitine exchange. PBC and PB-CoA exhibit excimer and monomer fluorescence, the relative intensities of which are functions of their microscopic concentrations. PB-CoA is accessible only to the outer half of artificial lipid vesicles while PBC may cross lipid vesicle bilayers. PBC in the inner half of the bilayer appears "trapped", i.e., not easily removed by exogenous bovine serum albumin, which binds PBC.

Spectroscopic investigations of the behavior of phospholipids, steroids, and free fatty acids in model membranes have led to a better understanding of the role of lipid constituents in biological membranes and the response of these lipids to various perturbations. In contrast, acyl coenzyme A (acyl-CoA) and acylcarnitine represent classes of amphipathic lipids of biophysical interest (Powell & Churchich, 1978; Devaux et al., 1975) that have been less extensively investigated. These molecules provide "activated" forms of free fatty acids to be used in cellular synthetic and oxidative pathways. Acyl-CoA is the biologically active form of free fatty acids used for phospholipid synthesis as well as for β-oxidation in the mitochondrial matrix. Acyl-CoA also interacts with a variety of soluble and membrane-bound proteins, in particular, the mitochondrial adenine nucleotide translocase (Pande & Blanchaer, 1971; Morel et al., 1974). Formation of acylcarnitine from long-chain acyl-CoA is necessary for the biological oxidation of fatty acids (Bremer, 1962). Both molecules have been shown to be inhibitors of a wide variety of membrane-associated pump mechanisms (McMillin-Wood et al., 1977) and have been postulated to be significant in the etiology of myocardial ischemia (McMillin-Wood et al., 1973). While these classes of molecules are biologically important, there is insufficient information concerning their membrane interactions to allow a complete understanding of their biological function.

In fluorescence studies of membrane systems, pyrene and its derivatives have been used to study bilayer–solvent properties, lateral and transverse diffusion rates, and intervesicular lipid transfer (Galla & Hartmann, 1980; Doody et al., 1980; Roseman & Thompson, 1980). The ability of pyrene to produce excimer fluorescence in a bimolecular diffusion-limited reaction (Forster & Kasper, 1955) makes it a useful probe in these types of studies. The origin of excimer fluorescence has been described elsewhere (Birks, 1970).

In this paper the synthesis of the carnitine and coenzyme A derivatives of 1-pyrenebutyric acid, a medium-chain fatty acid analogue, is described. These molecules have the expected characteristics of pyrene derivatives while retaining the biochemical character of their natural analogues. The specific interaction of these derivatives with mitochondrial membranes suggests their utility in elucidation of metabolic mechanisms.

Materials and Methods

Synthesis of Derivatives. 1-Pyrenebutyric acid (PBA) was purchased from Eastman Kodak, Inc. Dicyclohexylcarbodiimide (DCC) and carnitine were purchased from Tridom Fluka, CoA was purchased from Sigma Chemical Co., and 4-(1-pyrrolidinyl)pyridine was purchased from Aldrich Chemical Co. (1-Pyrenebutyryl)carnitine (PBC) and 1-pyrenebutyryl coenzyme A (PB-CoA) were synthesized according to the method of Patel et al. (1979), with minor modifications. PBA (190 mg) was suspended in 3–4 mL of freshly distilled tetrahydrofuran and 80 mg of DCC added. After 3–4 h the insoluble dicyclohexylurea was removed by gravity filtration. To this solution of 1-pyrenebutyryl anhydride was added either 80 mg of DL-carnitine or 250 mg of coenzyme A in warm Me_2SO,[1] previously dried over activated alumina and molecular sieves. 4-(1-Pyrrolidinyl)pyridine (40–50 mg) was added to the reaction mixture, which was placed under N_2, sealed, and allowed to react for 2–3 h.

PBC was purified according to the method of Bremer (1968). PB-CoA was purified in essentially the same manner except that the butanol washes were omitted and PB-CoA was separated from unreacted CoA by Sephadex LH-20 chro-

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[1] Abbreviations: Me_2SO, dimethyl sulfoxide; ^1H NMR, proton nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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matography with 0.003 N HCl, 0.003 N HCl–EtOH (1:1), and EtOH as a step gradient. PB-CoA eluted in the final solvent, free from unreacted CoA. Both derivatives were crystallized according to Ziegler et al. (1967). The acylcarnitine gave a microcrystalline product while the coenzyme A derivative gave larger crystals. The yields for both these reactions varied from 40 to 70%.

The ^1H NMR spectrum of PBC in perdeuteriomethanol was obtained by using a Varian EM 360 60-MHz spectrometer with tetramethylsilane as a reference. Integration and comparison of the carnitine choline signal (3.1 ppm) with the pyrene ring (7.6–8.2 ppm) gave a ratio of 1.06. Carnitine ester content, analyzed according to Idell-Wenger et al. (1978) and compared to pyrene absorbance (ϵ 40 000 M^{-1} at ca. 340 nm) (Knopp & Weber, 1969), was 0.98. Thin-layer chromatography (TLC) of PBC, according to the method of Bremer (1968) for acylcarnitines, gave a single fluorescent spot with an R_f of 0.55.

Solubility limitations of PB-CoA prevented ^1H NMR analysis. The thiol ester bond content determined by using the recycling assay of McDougal & Dargar (1979) gave a purity of 98% (i.e., CoA thiol ester vs. pyrene absorption). TLC of PB-CoA by using the method of Pullman (1973) for CoA esters gave a single fluorescent spot with an R_f of 0.5.

Preparation of Vesicles. Asolectin (Association Concentrates, Woodside, NY) (50 mg) was placed in 1 mL of 10–20 mM Tris buffer under N_2, sealed, and sonicated to clarity in a bath-type sonicator (Sonicator Laboratory Supply Co., Hicksville, NY). The homogeneity of these vesicles was assessed by using Sepharose 4B column chromatography (2.5 × 40 cm) eluted with 10 mM Tris(hydroxymethyl)aminomethane, 100 mM NaCl, 1 mM EDTA, and 1 mM NaN_3, pH 7.4. This analysis showed a single peak of 280 nm absorbing material with an elution volume identical with that of single-walled vesicles (Huang & Thompson, 1974). In bovine serum albumin (BSA) titration experiments, Sepharose 4B chromatography was used to analyze the degree of probe separation from vesicles.

Mitochondria: Isolation and Associated Activities. Mitochondria were isolated from rat liver as previously described (Wolkowicz & McMillin-Wood, 1980). Experiments measuring PB-CoA inhibition of active respiration were performed in a Yellow Springs Instrument Model 53 oxygen monitor. The respiratory assay contained 7.5 mM succinate, 10 μg of rotenone, 5 mM $MgCl_2$, 12.5 mM potassium phosphate, 37.5 mM KCl, 2 mM EDTA, 100 mM sucrose, 5 mM Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], 15 mM glucose, and 20 units of hexokinase (P-L Biochemicals) with 2 mg of mitochondrial protein in a final volume of 2 mL. Phosphorylating respiration was initiated with varying subsaturating concentrations of adenosine 5′-diphosphate (ADP) over a range from 8 to 67 μM. Assessment of (1-pyrenebutyryl)carnitine effects on respiration was carried out as above, at saturating ADP concentrations in the presence of malate, with either 7.5 mM glutamate, 10 μM palmitoylcarnitine, or 67 μM octanoylcarnitine as the substrate.

The carnitine palmitoyl-CoA transferase "forward" reaction (palmitoyl-CoA → palmitoylcarnitine) and inhibition studies were performed on isolated liver mitochondria by the 5,5′-dithiobis(2-nitrobenzoic acid) method of Fritz et al. (1963), while the "reverse" carnitine palmitoyl-CoA transferase assay (palmitoylcarnitine → palmitoyl-CoA) was according to Hoppel & Tomec (1972). Assay of carnitine octanoyl-CoA transferase was carried out on detergent-solubilized mitochondria and the product, octanoylcarnitine, isolated as described by Solberg (1974). The properties of (1-pyrenebutyryl)carnitine with respect to the carnitine acyltransferase were assessed by using the isotope-exchange method of Solberg (1974).

Table I: Distribution Coefficients of (1-Pyrenebutyryl)carnitine and 1-Pyrenebutyryl Coenzyme A[a]

	K_D^M	ΔG (kcal/mol)
(1-pyrenebutyryl)carnitine		
octanol/water	99	−2.58
hexane/water	0.42	+0.515
1-pyrenebutyryl-CoA		
octanol/water	0.955	+0.027
hexane/water		

[a] Probe molecules were suspended in 2 mL of water at a concentration of 20 μM, and 2 mL of the organic phase was added. Following mixing and separation the aqueous phase was retrieved, and changes in concentration were determined by changes in absorption at 340 nm (ϵ 40 000 M^{-1}). K_D^M was determined from the corrected molar content of the two phases as determined by absorption changes. ΔG values were determined from the formula $\Delta G = RT \ln K_D$.

The carnitine-acylcarnitine translocase activity was measured according to the method of Halperin & Pande (1979), with liver mitochondria loaded with [^{14}C]carnitine (Amersham, Arlington Heights, IL; sp act. = 1378 dpm/nmol of L-carnitine). [^{14}C]Carnitine efflux was initiated by addition of either 5–40 μM L-palmitoylcarnitine or 1.4–9 mM L-carnitine at 4 °C in the presence and absence of (1-pyrenebutyryl)carnitine.

Physical Measurements. Absorption spectra were obtained from a Zeiss UV–vis spectrophotometer, Model PMQ II. Fluorescence spectra of these derivatives were recorded on an SLM Model 8000 digital single photon counting spectrofluorometer with 320 nm as the exciting wavelength and with 2-nm slit widths. Static fluorescence measurements were obtained from an Aminco-Bowman spectrofluorometer with 320 nm as the exciting wavelength and 2-nm slit widths.

Partition coefficients between organic–water phases were measured by measuring the loss of pyrene absorption in the aqueous phase following mixing and separation of the two solvents. K_D values presented were calculated from $K_D = X(\text{organic})/X(H_2O)$ where X equals the mole fraction of the probe in the appropriate solvent.

Determination of the critical micelle concentration (cmc) of the pyrene derivatives was based on formation of excimer fluorescence as described by Galla et al. (1979). Monomer and excimer fluorescence was measured on a Farrand Mark IV spectrofluorometer with a 320-nm excitation wavelength of 2-nm slits.

BSA titration experiments were performed with Sigma fraction V albumin. Other reagents or chemicals used were of general laboratory grade.

Results and Discussion

In dilute solution the absorption and monomer fluorescence spectra of both derivatives were typical of pyrene-containing molecules (Figure 1). At higher probe concentrations a broad emission band centered at 475 nm appeared; this band was assigned to the well-known excimer fluorescence that has been previously identified for pyrene and some of its derivatives (Birks, 1970). In the case of the CoA derivative the absorption spectrum was distorted in the UV range of 230–289 nm due to the CoA moiety itself and the thiol ester bond.

The distribution coefficients of the two derivatives between water and octanol or hexane are shown in Table I. In contrast

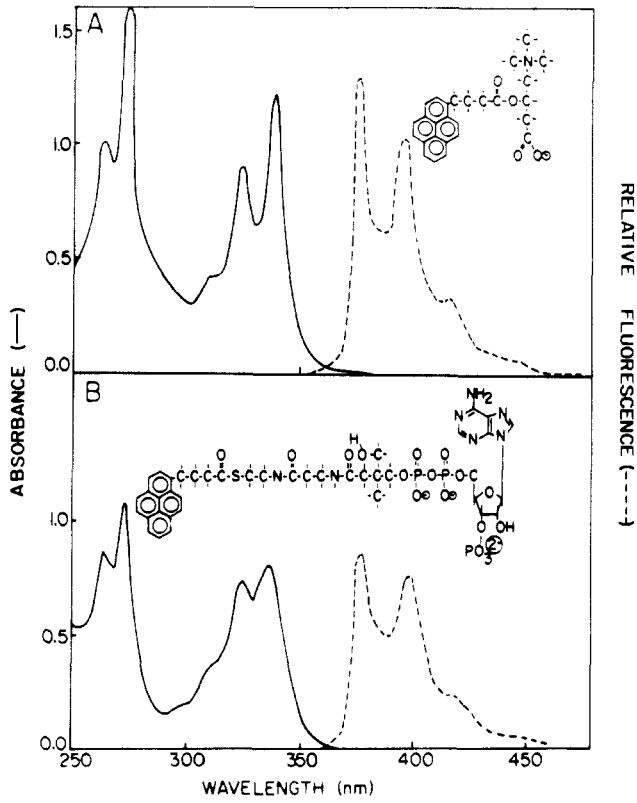

FIGURE 1: (A) Structural formula and absorption (—) and fluorescence (- - -) spectra of 30 μM (1-pyrenebutyryl)carnitine at pH 7.0. (B) Structural formula and absorption (—) and fluorescence (- - -) spectra of 19 μM 1-pyrenebutyryl coenzyme A at pH 7.0.

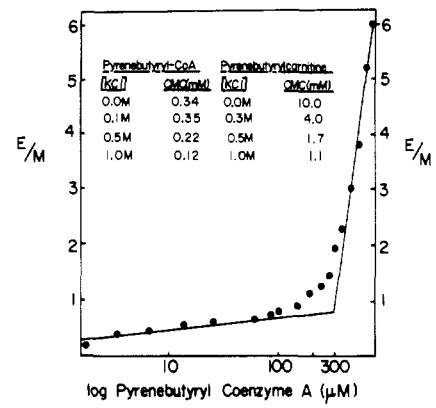

FIGURE 2: Critical micelle concentrations of 1-pyrenebutyryl-CoA and (1-pyrenebutyryl)carnitine determined from pyrene excimer/monomer (E/M) fluorescence. All determinations were carried out at pH 7.4 in the absence and presence of varying concentrations of KCl. An abrupt increase in excimer fluorescence indicates formation of micellar aggregates in solution. (Inset) Summary of critical micelle concentrations (cmc, mM) for 1-pyrenebutyryl-CoA and (1-pyrenebutyryl)carnitine at varying concentrations of KCl.

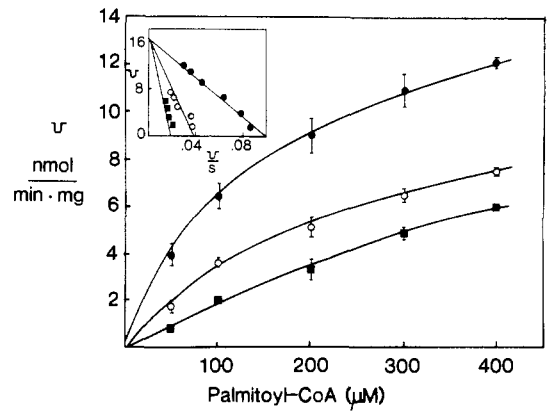

FIGURE 3: 1-Pyrenebutyryl coenzyme A inhibition of carnitine palmitoyl-CoA transferase in rat liver mitochondria. The forward reaction was measured with 1–2 mg of mitochondrial protein by using the 5,5′-dithiobis(2-nitrobenzoic acid) method of Fritz et al. (1963). 1-Pyrenebutyryl-CoA at 4 (○) or 8 μM (■) was used to inhibit conversion of palmitoyl-CoA to palmitoylcarnitine [control rates (●)]. Results are the average of four separate experiments ± standard error of the mean.

with the marked solubility of (1-pyrenebutyryl)carnitine in octanol, the CoA derivative was not exceedingly soluble in this solvent. The carnitine derivative had limited solubility in hexane while the CoA derivative was essentially insoluble. In the case of the carnitine derivative in either organic solvent the molecules exist essentially in the monomer form (i.e., I_E/I_M of the fluorescence was very low), while in the case of 1-pyrenebutyryl-CoA the observation of a high excimer to monomer ratio in the octanol phase indicated the presence of aggregate forms of the probe molecules, possibly as reverse micelles.

The critical micelle concentrations (cmc) of 1-pyrenebutyryl-CoA and (1-pyrenebutyryl)carnitine at low salt concentrations and the decrease in the respective cmc values in the presence of KCl (Figure 2) are similar to values reported for medium-chain acyl esters (Yalkowsky & Zografi, 1970). These data suggest that the pyrene derivatives are physical analogues of medium-chain acyl-CoA and acylcarnitine.

Fatty acyl-CoA in the absence of carnitine inhibits ADP-stimulated mitochondrial respiration due to inhibition of adenine nucleotide translocase activity. 1-Pyrenebutyryl-CoA mimicked this property of the natural metabolites. 1-Pyrenebutyryl-CoA was a noncompetitive inhibitor of rat liver mitochondrial phosphorylating (ADP-stimulated) respiration with a K_I of 2 μM. 1-Pyrenebutyryl-CoA exerted no effect on the hexokinase enzyme system at the concentrations used. Since the 1-pyrenebutyryl-CoA derivative appears to be a medium-chain fatty acid analogue, the low K_I and inhibition pattern suggest that the pyrene ring structure may play a role in binding to the translocase. (1-Pyrenebutyryl)carnitine had no effects on the kinetics of ADP-stimulated respiration with succinate (or glutamate) as the respiratory substrate.

Fatty acyl-CoA is the physiological substrate for the carnitine acyltransferase(s) of the inner mitochondrial membrane. 1-Pyrenebutyryl-CoA in the presence of carnitine was not a substrate for carnitine acyltransferase catalysis as judged by the absence of free CoA generation (Fritz et al., 1963) within the time frame (10 min) and concentration (100 μM) used. However, 1-pyrenebutyryl-CoA was a competitive inhibitor toward palmitoyl-CoA in the carnitine palmitoyl-CoA transferase forward reaction (K_I = 2.1 μM; Figure 3). These experiments were conducted with excess L-carnitine to avoid complications regarding acyl-CoA inhibition of carnitine binding as described by Bremer & Norum (1967).

Although octanoate is not dependent on carnitine for its oxidation (Fritz, 1959), the existence of a carnitine octanoyltransferase has been proposed in liver mitochondria (Solberg, 1974). However, medium-chain transferase activity may reflect differing properties of the purified beef heart carnitine palmitoyltransferase (Clarke & Bieber, 1981). 1-Pyrenebutyryl-CoA was also found to be a competitive inhibitor of this medium-chain transferase activity by using octanoyl-CoA as the substrate in the presence of carnitine. However, the K_I was 7-fold higher (14.5 μM) than the observed K_I for inhibition of carnitine palmitoyltransferase. As in the case of the inhibition kinetics of the adenine nucleotide

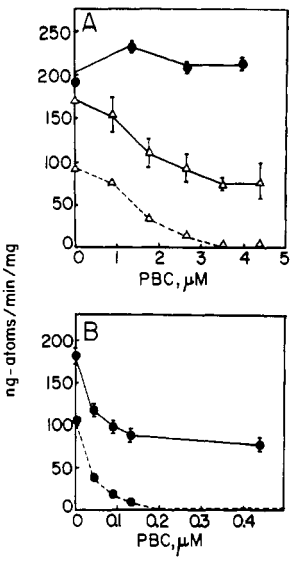

FIGURE 4: (A) Effect of (1-pyrenebutyryl)carnitine on palmitoylcarnitine-supported rat liver mitochondrial respiration. In control experiments (●) 7.5 mM glutamate, 3 mM malate, and 10 μM palmitoylcarnitine were present as respiratory substrates. In other experiments, 3 mM malate and 10 μM palmitoylcarnitine were substrates (Δ—Δ). The inhibition of respiration was corrected for contribution of malate respiration to these rates (Δ---Δ). Results represent four different experiments ± standard error of the mean. (B) Effect of (1-pyrenebutyryl)carnitine on octanoylcarnitine-supported respiration in rat liver mitochondria. Control experiments (●—●) utilized 62 μM octanoylcarnitine and 3 mM malate as substrates. These rates of respiration were corrected for malate respiration (●---●) at varying probe concentrations. Results represent four different experiments ± standard error of the mean.

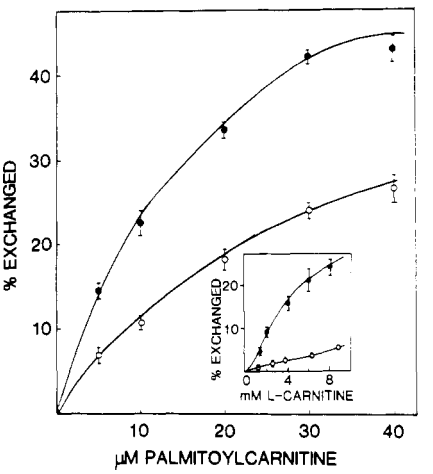

FIGURE 5: Inhibition of carnitine–palmitoylcarnitine exchange in rat liver mitochondria by (1-pyrenebutyryl)carnitine. Rat liver mitochondria were loaded with [^{14}C]carnitine as described by Halperin & Pande (1979). Efflux of labeled carnitine was followed upon addition of L-palmitoylcarnitine at 1 min at 0 °C by the mersalyl-stop technique. When 1 μM (1-pyrenebutyryl)carnitine was present (○), efflux of labeled carnitine upon addition of L-palmitoylcarnitine was measured at 3 min at 0 °C with mersalyl to inhibit further exchange. Control rates of exchange (●) are the average of five determinations ± standard error of the mean. (Inset) Inhibition of carnitine–carnitine exchange in rat liver mitochondria by (1-pyrenebutyryl)carnitine. Rat liver mitochondria [^{14}C]carnitine efflux was followed as described above upon addition of unlabeled L-carnitine. When 1 μM (1-pyrenebutyryl)carnitine was present, efflux of labeled carnitine was measured at 3 min at 0 °C (mersalyl-stop technique). Control rates of exchange (●) and rates in the presence of 1 μM PBC (○) are the average of six determinations ± standard error of the mean.

translocase by 1-pyrenebutyryl-CoA, the molecular features of the pyrene ring may produce an altered interaction of inhibitor at the fatty acyl-CoA site of the acyltransferase(s) than would be anticipated from its medium chain length solution properties.

(1-Pyrenebutyryl)carnitine was not a substrate for the carnitine acyltransferase where acyl[^{14}C]carnitine formation in the presence of CoA was measured by the isotope-exchange assay (Solberg, 1974) and had no effect upon either the forward or reverse reaction kinetics of carnitine palmitoyl-CoA transferase.

(1-Pyrenebutyryl)carnitine has no effect on ADP-stimulated respiration with succinate (+rotenone), glutamate–malate, or malate alone as the substrate. Control respiratory rates with glutamate–malate and varying concentrations of (1-pyrenebutyryl)carnitine were determined in the presence of 10 μM palmitoylcarnitine to assure that any changes in substrate oxidation could not be due to nonspecific effects, e.g., swelling, on the mitochondria (Figure 4A, upper curve). However, when palmitoylcarnitine (10 μM) in the presence of malate was the sole oxidative substrate, (1-pyrenebutyryl)carnitine produced a concentration-dependent inhibition of state-3 respiration (Figure 4A, middle curve). Correction of these rates for malate oxidation is shown in the lower curve of Figure 4A. An approximate I_{50} value of 1.45 μM for (1-pyrenebutyryl)-carnitine inhibition can be estimated. Although the active respiratory rates of malate oxidation are most likely to be elevated during palmitoylcarnitine oxidation, the overall shape of the inhibition curve should not be significantly altered.

When octanoylcarnitine was the respiratory substrate in the presence of malate, (1-pyrenebutyryl)carnitine had a dramatic inhibitory effect on phosphorylating respiration (Figure 4B, upper curve). After correction for malate oxidation, an I_{50} for inhibition of octanoylcarnitine respiration was estimated to be 40 nM (Figure 4B, lower curve).

Since (1-pyrenebutyryl)carnitine at these concentrations had no inhibitory effects on the carnitine acyltransferase enzymes, it seemed reasonable that this inhibitor might affect entry of fatty acylcarnitine into the mitochondria via the carnitine–acylcarnitine translocase. Therefore, the effect of (1-pyrenebutyryl)carnitine on exchange of radioactive carnitine from [^{14}C]carnitine-loaded mitochondria in the presence of L-palmitoylcarnitine or L-carnitine was measured as described by Halperin & Pande (1979). When palmitoylcarnitine was the substrate for the translocase, 1 μM (1-pyrenebutyryl)carnitine significantly reduced the back-exchange of [^{14}C]carnitine (Figure 5). Conversion of exchange rates to velocity (Halperin & Pande, 1979) and kinetic analysis of the data gave a K_I for (1-pyrenebutyryl)carnitine inhibition of palmitoylcarnitine–carnitine exchange of 0.58 μM.

Attempts to follow DL-octanoylcarnitine translocation produced erratic results due to the presence of the D isomer (Pande & Parvin, 1980). When L-carnitine was employed as a substrate for the translocase, exchange was potently inhibited by 1 μM (1-pyrenebutyryl)carnitine (Figure 5, inset), and a K_I for inhibition of carnitine translocation was calculated from the slopes of the double-reciprocal plot (r = 0.9997) to be 23 nM. These results were in agreement with the I_{50} ranges approximated from the respiration studies (Figure 4) and indicate a specific interaction of (1-pyrenebutyryl)carnitine with the mitochondrial carnitine–acylcarnitine translocase.

Since 1-pyrenebutyryl-CoA and (1-pyrenebutyryl)carnitine demonstrated significant and specific interactions with mitochondrial membrane-associated processes, the property of pyrene excimer fluorescence was employed to investigate the association of these derivatives as well as 1-pyrenebutyric acid with artificial membranes. The ratio of excimer to monomer fluorescence for these molecules increases linearly over a probe concentration range of 0.2–1.5 mol %, in the presence of

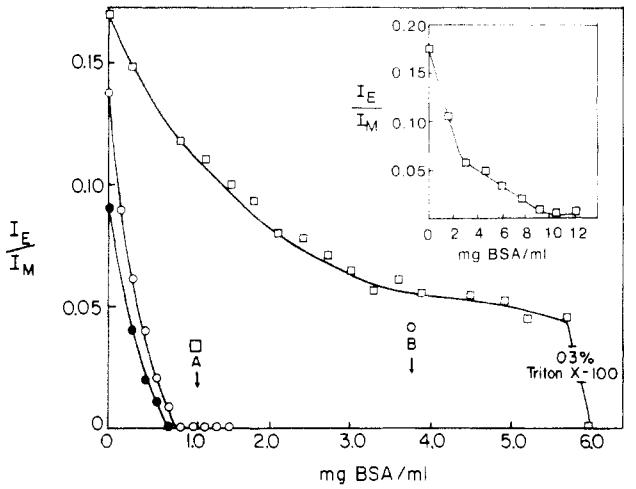

FIGURE 6: BSA titration of asolectin vesicles containing various probe molecules. Asolectin vesicles (0.5 mg/mL), prepared as described under Materials and Methods, were doped by exogenous probe addition of either (1-pyrenebutyryl)carnitine (□) or 1-pyrenebutyryl-CoA (○) or by sonication of asolectin (50 mg) in the presence of 1 mol % 1-pyrenebutyric acid (●). In all experiments the initial concentration of single-walled vesicles (Huang & Thompson, 1974) was 0.5 mg/mL. Varying concentrations of BSA (Sigma fraction V) were added to the reaction mixture as indicated, and I_E/I_M was determined within 30 s thereafter. Triton X-100 (0.3% final concentration) was added where indicated in the (1-pyrenebutyryl)carnitine–BSA titration curve. Arrows indicated by A and B represent 1 mol of PBC/mol of BSA and 1 mol of PB-CoA/mol of BSA, respectively. (Inset) BSA concentrations were increased to 12 mg/mL during exogenous PBC titration.

asolectin vesicles (data not shown). This behavior reflects an increase in microscopic probe concentration in the lipid bilayer.

The limited water solubility of 1-pyrenebutyric acid required its cosonication into lipid vesicles, whereas 1-pyrenebutyryl-CoA and (1-pyrenebutyryl)carnitine could be incorporated (exogenously) by simple addition, or alternatively, both derivatives could be cosonicated with the lipids during vesicle preparation. Vesicles containing these molecules (i.e., 1-pyrenebutyric acid cosonicate, 1-pyrenebutyryl-CoA, or (1-pyrenebutyryl)carnitine added exogenously) were then titrated with fatty acid free bovine serum albumin (BSA), which binds all three types of molecules (Figure 6).

Since the free acid of acylpyrene undergoes rapid transbilayer diffusion (Galla et al., 1979; Doody et al., 1980), addition of BSA to vesicles doped with 1-pyrenebutyric acid produced a decrease in the observed I_E/I_M to its zero value (Figure 6), indicating removal of 1-pyrenebutyric acid from the microenvironment of the lipid bilayer. Likewise, the CoA derivative added exogenously to preformed vesicles was removed by increasing amounts of BSA as indicated by I_E/I_M measurements (Figure 6). In data not shown, the I_E/I_M ratio of 1-pyrenebutyryl-CoA cosonicated into vesicles (i.e., present in both bilayer halves) could only be lowered to a value 20–30% of the original by addition of BSA. Excess BSA does not affect this result, but addition of detergent (0.3% Triton X-100) to disrupt vesicle integrity reduces the I_E/I_M to its zero value.

When (1-pyrenebutyryl)carnitine was added exogenously to vesicles (Figure 6) or cosonicated (data not shown), low concentrations of BSA decreased the I_E/I_M ratio to a nonzero value that was 25% of the original I_E/I_M. Addition of 0.3% Triton X-100 (Figure 6) or higher concentrations of BSA (Figure 6, inset) decreased the I_E/I_M value for (1-pyrenebutyryl)carnitine to zero in both cases (cosonicated or exogenously added). These results suggest that the carnitine derivative is associated with both sides of the vesicle bilayer in each case (exogenous or cosonicated). As opposed to 1-pyrenebutyryl-CoA, exogenous addition of (1-pyrenebutyryl)carnitine results in a portion of the excimer fluorescence that was not easily accessible to BSA, indicating that the latter compound crosses the lipid membrane but that its back-diffusion is restricted (compare to the free acid).

Vesicle solutions prepared under the various conditions described were analyzed with Sepharose 4-B chromatography, which separates the pyrene derivatives that are free in solution from the derivatives that are associated with the vesicles. In the absence of BSA, exogenous 1-pyrenebutyryl-CoA was both

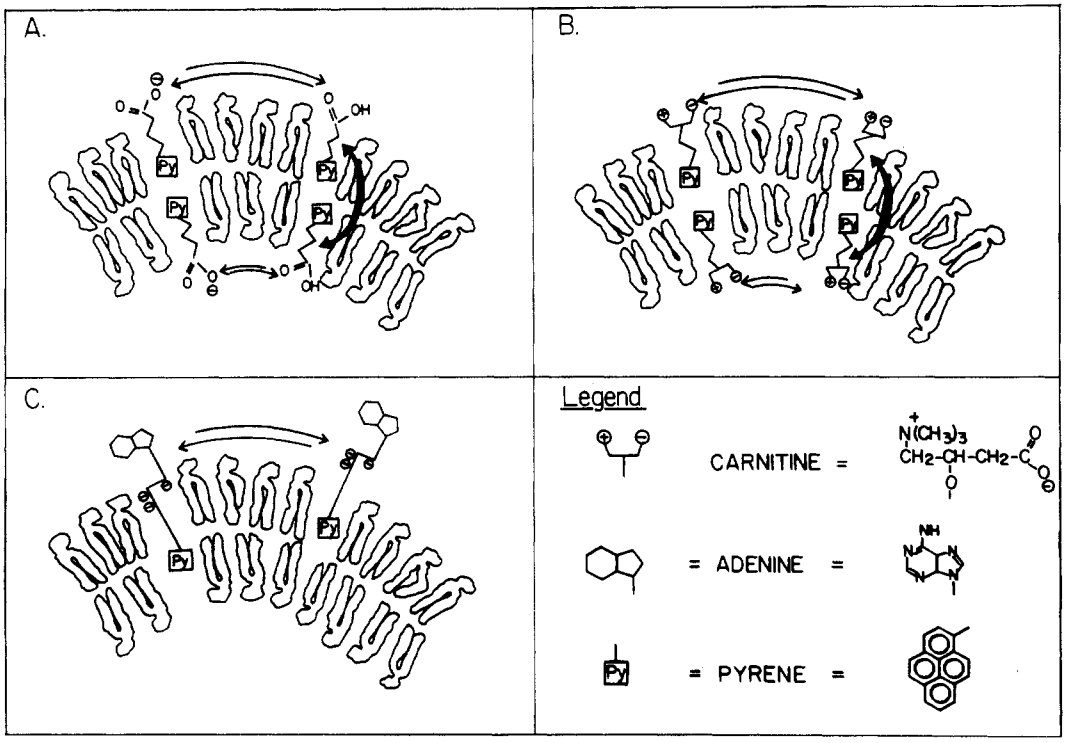

FIGURE 7: Schematic representation of proposed membrane–probe interaction. (A) 1-Pyrenebutyric acid; (B) (1-pyrenebutyryl)carnitine; (C) 1-pyrenebutyryl-CoA.

free in solution and bound to vesicles while the majority of (1-pyrenebutyryl)carnitine was vesicle bound. Addition of BSA at appropriate concentrations showed that all of the exogenous CoA derivative was removed from the vesicles while a substantial portion of the (1-pyrenebutyryl)carnitine was still membrane bound, thereby supporting the interpretation of the I_E/I_M measurements.

Exogenous BSA should sequester available probe molecules, and as BSA is titrated into the various experimental systems, the perturbed equilibrium should cause a decrease in vesicle-bound probe molecules and a consequent decrease in I_E/I_M. This pattern holds in the case of the free acid and the exogenously added 1-pyrenebutyryl-CoA, indicating complete availability to BSA. However, exogenously added or cosonicated (1-pyrenebutyryl)carnitine and cosonicated 1-pyrenebutyryl-CoA show two types of distinct and extended plateaus in the BSA titration curve. This suggests that these probes have entered into a compartment that is inaccessible or only slowly accessible to equilibrium binding by BSA, i.e., a space from which exit is not kinetically or thermodynamically favorable (as is the case for the exogenous CoA derivative or the reverse flip-flop of the free acid). The main possibility for this "space" appears to be the inner monolayer of the vesicle. These data would indicate that in artificial lipid bilayers 1-pyrenebutyric acid is accessible to both sides of the bilayer and can freely translocate from one half to the other. The CoA derivative added exogenously is accessible to the outer face of the bilayer only, while the carnitine derivative is able to cross the bilayer.

We postulate that PB-CoA cosonicated into the inner half of the vesicle would be trapped and inaccessible to rapid removal by BSA. This is due to the size and ionic nature of the molecule, which limits its ability to undergo transbilayer diffusion. In contrast, PBC may traverse the membrane by an ion paired state translocation mechanism described by Murray et al. (1980). Inside the vesicle lumen, the increase in solvent polarity (Clement & Gould, 1980) would not favor the formation of the carnitine head-group ionic pair, thus decreasing the rate of exit of derivative from this face of vesicle (Figure 7). At present the interpretation given is speculative and further experiments are in progress to test these and other possibilities. A physiological consequence of this "trapping" of acylcarnitine could be postulated in the mitochondrion. This process would facilitate β-oxidation with regeneration of free carnitine for outward exchange. To our knowledge, exchange of matrix acylcarnitine with cytosolic free carnitine has not been described. In this context, Idell-Wenger et al. (1978) have demonstrated accumulation of long-chain acylcarnitines in mitochondria isolated from ischemic hearts. These results as well as the present experiments suggest that acylcarnitine trapping in the appropriate ionic environment may regulate directionality of movement by a carrier or free diffusion mechanism.

In natural membrane systems such as the mitochondrial inner membrane, the free transbilayer movement of acylcarnitine may be restricted by the presence of integral membrane proteins (Conrad & Singer, 1981). The mechanisms by which acylcarnitines may traverse the mitochondrial inner membrane have been subject to several lines of speculation and experiment. Arguments exist for both carrier (Pande, 1975; Ramsay & Tubbs, 1975) and noncarrier (Levitsky & Skulachev, 1972; Murray et al., 1980) mediated mechanisms. The sensitivity of palmitoyl- and octanoylcarnitine-supported mitochondrial respiration to (1-pyrenebutyryl)carnitine [and the lack of effect of this derivative on the carnitine acyltransferase(s) of mitochondria] is strongly supportive of a specific-carrier mechanism. Moreover, the potent inhibition by (1-pyrenebutyryl)carnitine of mitochondrial matrix [^{14}C]carnitine exchange with either palmitoylcarnitine or free L-carnitine is further proof of this suggestion. Finally, the high specificity of this derivative for the carnitine translocase demonstrated by its low K_I and competitive kinetics with long-chain acylcarnitine, as well as free carnitine, and its lack of effect on other membrane-associated systems makes (1-pyrenebutyryl)carnitine a useful tool in the further understanding of the nature of this transport system.

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Polymerization of the Tubulin–Colchicine Complex and Guanosine 5′-Triphosphate Hydrolysis†

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ABSTRACT: The tubulin–colchicine (1:1) complex was shown to be able to polymerize in vitro under the buffer conditions of microtubule assembly from pure native tubulin. The physical characteristics of this peculiar polymer have been investigated under a variety of conditions and compared with those of microtubules. Polymerization consisted of a nucleation followed by a growth process, was characterized by a critical concentration, and exhibited divalent ion, temperature, and pH dependences very similar to those of microtubules. Guanosine 5′-triphosphate (GTP) or 5′-guanylyl methylenediphosphate (GMPPCP) was required for polymerization, and guanosine 5′-diphosphate (GDP) was a potent inhibitor. GTP hydrolysis was totally disconnected from the polymerization process and occurred as well under nonpolymerizing conditions. The results are discussed in view of the different types of protein–protein interactions exhibited by tubulin and of the possible relationship between the conformation of the GTP site and the interaction areas.

Substoichiometric amounts of colchicine are known to inhibit the microtubule assembly process in vivo as well as in vitro (Taylor, 1965; Olmsted & Borisy, 1973; Wilson et al., 1975). Colchicine binds to tubulin with a high affinity (Bryan, 1972; McClure & Paulson, 1977), and the quasi-irreversible binding permits the isolation of the tubulin–colchicine complex and its use as an excellent tool in the investigation of the mechanism of assembly via inhibition studies. In this respect, the following points have been established: Two types of tubulin–tubulin interactions are involved in the microtubule wall, namely, strong longitudinal interactions along protofilaments and weaker lateral interactions (Erickson, 1974; Amos & Baker, 1979; Erickson & Pantaloni, 1981). While colchicine does not bind to tubulin once incorporated in the microtubule (Wilson & Meza, 1973), except to the ends (Margolis & Wilson, 1977), the colchicine site is still accessible on linear polymers such as rings (Weisenberg & Timasheff, 1970; Penningroth, 1980) in which only longitudinal interactions between tubulin molecules are involved. Recently Sternlicht & Ringel (1979) reported the possibility of a very slight incorporation of tubulin–colchicine in the microtubule; this copolymerization would then be made possible pending a destabilization of the microtubules in proportion with the amount of tubulin–colchicine incorporated. All these data suggested that the colchicine site is close to one of the two lateral interaction areas of tubulin (David-Pfeuty et al., 1979). Furthermore, the findings that a GTPase activity is induced on tubulin by colchicine binding (David-Pfeuty et al., 1979) and that GTP[1] is hydrolyzed on microtubules in a first-order process subsequent to tubulin assembly (Carlier & Pantaloni, 1978) are suggestive of a connection between the conformation of one of the two lateral interaction areas and the behavior of the GTP exchangeable site, which is located on the β subunit (Geahlen & Haley, 1979). In this hypothesis, both the GTP site and the colchicine site, which genetic studies indicate to be also located on the β subunit (Cabral et al., 1981; Sheir-Neiss et al., 1978), would be in a close vicinity to the lateral interaction area of the β subunit, which seems bound to an α subunit of the adjacent protofilament by a loose protein bridge observable on electron micrographs (Amos & Klug, 1974; Erickson, 1974).

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine 5′-triphosphate; DEAE, diethylaminoethyl; NTP, unspecified nucleoside 5′-triphosphate; GMPPCP, 5′-guanylyl methylenediphosphate.

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