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Interaction of amphiphilic substrates (acyl-CoAs) and their metabolites (free fatty acids) with microsomes from mouse sciatic nerves

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We have measured the partition of stearoyl-CoA and oleoyl-CoA between an aqueous phase and the microsomes from mouse sciatic nerves. A method of microultracentrifugation was used which allowed us to study separately the aqueous phase and the biological membranes. We observed that the partition is dependent upon the amount of acyl-CoAs and membrane proteins but seems to be independent of time. A theoretical analysis of these data allowed interpretation of the binding and release in terms of acyl-CoA surface density in the vesicles. We have also analyzed the fate of the membrane-bound acyl-CoAs. We show that, whereas the apparent partition does not seem to vary, the hydrolysis of the membrane-bound acyl-CoAs followed by the release of free fatty acids from the membrane leads to a modification of the partition of acyl-CoAs between the membrane and the aqueous phase. We propose that there is a constant partition of the aliphatic chains (acyl-CoAs + free fatty acids).

Long chain acyl-CoAs, substrates of many membrane-bound enzymes, have to interact with the membrane prior to or during their metabolism. Therefore numerous studies have been carried out to analyze the partition of acyl-CoA (and free fatty acids) into lipid vesicles, used as model membranes (see, for example, Refs. 1 and 2). The binding of acyl-CoA and/or free fatty acids to proteins like BSA or FABP has also been considered [3] and in some cases, the binding and the partition of fatty acids in the presence of both binding proteins and lipid bilayers in the incubation mixture has been examined [4–7]. However, the use of artificial phospholipid vesicles did not allow, of course, the study of the metabolism of the membrane-bound substrates.

On the other hand, numerous studies concerning the metabolism of acyl-CoA by a biological membrane have been reported. In most cases, the complexity of the reactions catalyzed by membrane-bound enzymes using amphiphilic substrates has been overlooked and these reactions have been mostly considered as reactions catalyzed by soluble enzymes using soluble substrates. In fact, as membrane-bound enzymes use generally membrane-bound amphiphilic substrates, the kinetic parameters are strictly dependent upon the local (membrane) availability of the substrates, which has to be determined [8]. This type of analysis was recently performed: Noy and Zakim [9], Daniels et al. [4] and Pauly and Mac Millin [10] have analyzed the metabolism of acyl-CoA considering their local (i.e., membrane-bound) concentration. These studies did not take into account the effect of metabolism of membrane-bound substrates on the distribution of acyl-CoAs (and their metabolites) between the membrane and the aqueous phase. The literature reports evidences of either the partition of acyl-CoAs between an aqueous phase and membranes or their metabolism, without clear link between these two important phenomena. If it is easy to accept that the apparent partition of amphiphilic substrates is a composite of the amount that is bound and the amount that has undergone the metabolism, it is extremely difficult to determine the contribution of metabolism to the general process. The goal of this paper is to investigate

Abbreviations: PtdCho, phosphatidylcholine; FABP, fatty acid binding protein; FFA, free fatty acid; HPTLC, high performance thin-layer chromatography; CMC, critical micellar concentration.

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how metabolism can influence the partition of acyl-CoAs between membranes and the aqueous phase.

We have shown previously that in microsomes from mouse sciatic nerves, three main reactions need acyl-CoAs, namely the reactions catalyzed by acyl-CoA elongases [11] acyl-CoA thioesterase [12] and acyl-CoA transacylase [13]. In the absence of exogenous malonyl-CoA and NADPH, no elongation of the substrate may occur, but the hydrolysis of the substrate takes place as long as no ATP is added to the reaction mixture. BSA may also abolish free fatty acid release, but in this case, the binding of the substrate to the membrane could be considerably lowered because of the competitive binding of acyl-CoA with the BSA [10]. The third important activity is that of the acyl-CoA transacylase(s) which may catalyse acyl transfer from acyl-CoA to an exogenous lysolipid.

In order to minimize the complexity of the system under study, we omitted malonyl-CoA, NADPH and exogenous acyl acceptors from the reaction mixture, so that, in theory only the hydrolysis of acyl-CoA could take place, and we have investigated the contribution of this hydrolysis of acyl-CoA to the binding and/or release of acyl-CoA to/from the membrane.

The studies have been carried out as a function of both the amount and concentration of acyl-CoA, amount of microsomal membrane and as a function of time. The influence of a protein (BSA) able to bind the acyl-CoA in the aqueous phase has been determined. The release of these molecules from biological membranes (and not only their binding to these membranes) has also been studied. By comparing the partition of oleoyl-CoA for native and heat-inactivated membranes, we have determined the effect of acyl-CoA hydrolysis (and free fatty acids release) on this partition. This allowed us to propose a model able to interpret the experimental data.

Experimental procedures

Chemicals

Radioactive [1 – ^{14}C]stearoyl-CoA (55 mCi/mmol), and [1 – ^{14}C] oleoyl-CoA (52 mCi/mmol) were from Amersham, Les Ulis, France. Solvents were purchased from Merck, France. All other reagents were from Sigma Chemical Co (St Louis, MO, U.S.A.).

Preparation of mouse sciatic nerve microsomes

Mice, originally obtained from the B6-CBA strain, were bred in our Institute. Sciatic nerves of mice were removed and homogenized in a glass tissue grinder with a 0.05 M Tris-HCl buffer (pH 7.5) at 4 °C. The homogenate was spun at 20000 × *g* for 20 min. The 20000 × *g* supernatant was centrifuged at 150000 × *g* for 90 min. The 150000 × *g* pellet was resuspended in 200 μl of the same buffer. Heat-trated microsomes were let for 30 min at 60 °C before use. Proteins were estimated by the method of Bradford [14].

Partition of amphiphilic substrates

The study of acyl-CoA binding was carried out in a final volume of 50 μl in 0.05 M Tris-HCl (pH 7.5) at 37 °C (in some cases a different volume was used as described in the Results). Routinely, reactions were initiated by 10 μg of microsomal proteins; after 30 min, the reaction mixture was centrifuged with a Beckman TL 100, at 184000 × *g* for 10 min and yielded a membrane pellet (P1) and a supernatant (S1). The P1 pellet was resuspended in 50 μl of 0.05 M Tris-HCl (pH 7.5 0. The radioactivity of P1 and S1 was measured in a Packard 2000 CA liquid scintillation counter after addition of 2 ml of liquid scintillation cocktail (Opti-Fluor) to 5 μl of P1 or S1. The partition of acyl-CoA was also studied in the presence of 50 μg BSA in the initial reaction mixture.

Lipid analysis

Aliquots of the different fractions were mixed with 6 vol. of $CHCl_3/CH_3OH$ (1:1, v:v). Aliquots of the homogeneous phase obtained were loaded on 10 × 10 cm HPTLC Kieselgel 60 plates (Merck) and eluted according to Vitiello and Zanetta [15]: methyl acetate/*n*-propanol/ chloroform/ methanol/0.25% aqueous KCl (25:25:25:10:9), to separate acyl-CoAs, 1-acyl-GPC, PtdCho and free fatty acids. Each of the 6 lanes on the plate was 8-mm wide and separated from each other by 7 mm. The 6th lane was used for standardization with labeled lipids.

After elution, autoradiographs were prepared (Kodak DEF 5) to identify the lipids; the different lipid bands were scraped off directly into a scintillation vial and their radioactivities were determined.

Partition of acyl-CoAs after addition of CoASH

10.5 μg microsomes were (or not) preincubated with 0.5 nmol CoA, and then incubated for 30 min with 0.33 nmol or 0.66 nmol [1 – ^{14}C]oleoyl-CoA in a final volume of 50 μl. The P1 and S1 were prepared as described earlier and the distribution of the radioactivity was analyzed.

Release of acyl-CoAs

The membrane pellet (P1) was homogenised in 50 μl 0.05 M Tris-HCl (pH 7.5) and spun down again by microultracentrifugation to yield a second membrane pellet (P2) and an aqueous phase (S2). The label associated with P2 and S2 was determined; the lipids from P2 and S2 were analyzed by HPTLC and their radioactivities determined as described above.

Results

Partition of the acyl-CoAs

The partition of a constant amount (0.5 nmol) of [1 $-^{14}$C]stearoyl-CoA (C18-CoA) and of [1 $-^{14}$C]oleoyl-CoA (C18:1- CoA) between a constant volume of aqueous phase and an increasing amount of microsomes (estimated by the assay of microsomal proteins) is given in Fig. 1. The binding of labeled molecules increases as a function of the amount of microsomal proteins: 40% of the label are bound to 5 μg proteins, and a plateau is reached for 50 μg proteins, when 85 to 90% of the initial oleoyl-CoA are bound to the membranes. Very similar results are obtained with the saturated stearoyl-CoA (Fig. 1), and in this case, more than 80% of the labeled molecules are bound to the membranes when 0.5 nmol C18-CoA are added to 40 μg membrane proteins in a final volume of 0.1 ml at 37 °C.

The binding of labeled molecules increases almost linearly as a function of the amount of acyl-CoAs added to a constant amount of microsomes (Fig. 2). When 10 μg microsomal proteins are used, no saturation of the binding is observed whatever the amount of acyl-CoA, up to 6 nmol. These results are in good agreement with those of Pauly and McMillin [10], the long chain acyl-CoAs bind easily to the membrane so that the concentration parameter may reach 200 to 300 nmol bound/mg of microsomes. However, in the case of microsomes from mouse sciatic nerves, no deviation of the curve at high levels of added acyl-CoAs was observed, suggesting no saturation of the membrane

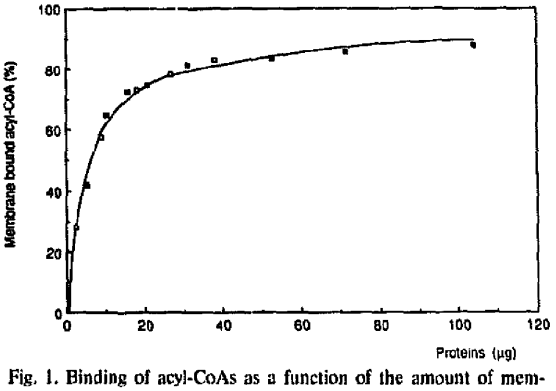


Fig. 1. Binding of acyl-CoAs as a function of the amount of membrane protein. 0.5 nmol of labeled acyl-CoA were incubated with various amounts of mouse sciatic nerves microsomes for 30 min at 37 °C in 50 μl 0.05 M Tris-HCl buffer (pH 7.5). The membrane bound radioactivity was determined as described in Experimental procedures. Results are expressed as percent of administered [1 $-^{14}$C]acyl-CoA. □, C18-CoA (final volume 100 μl); ■, C18:1-CoA (final volume 50 μl).

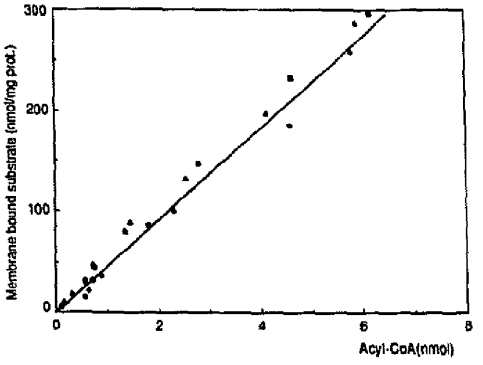


Fig. 2. Binding of acyl-CoAs as a function of the amount of acyl-CoA. Various amounts of labeled stearoyl-CoA (open symbols) or oleoyl-CoA (closed symbols) were incubated for 30 min at 37 °C with 10 μg of mouse sciatic nerves microsomes in 0.05 M Tris-[IC] buffer (pH 7.5); final volume 50 μl: ▲; 100 μl: ◆ or ◇; 300 μl: ■. The membrane bound radioactivity was determined as described in Experimental procedures.

with the acyl-CoA and no solubilization of membrane proteins during the studies of binding.

The study of the label partition into native membranes was also carried out in a final volume of 100 or 300 μl instead of 50 μl. As seen in Fig. 2, no difference in the binding was observed when the volume was increased suggesting that the amount but not the concentration of the acyl-CoAs plays a role in the binding to a constant amount of membrane. This is in fairly close agreement with our previous proposal that the true parameter to be considered is the ratio of the amount of acyl-CoA (nmol) to membrane proteins (μg) [13].

Under the experimental conditions described in Fig. 2, we studied the binding of the radioactivity as a function of time. To a constant amount of microsomes (10 μg proteins) from mouse sciatic nerves, constant amounts (0.5 nmol) of labeled oleoyl-CoA were added. After various times of incubation (2; 5; 10; 20; 30 and 60 min) the membranes were spun down by microultracentrifugation. The label associated with the membrane did not change greatly as a function of time and accounted for 57.7% ± 5.3% of the total added to the reaction mixture over a 60 min. period (Fig. 3, inset).

The partition of increasing amounts of acyl-CoA was not also greatly modified when the microsomes were heat-inactivated at 60 °C for 30 min (fig 3); Comparing the results obtained with native and heat treated microsomes, the mean slope of the curves dropped from 0.59 to 0.51.

From the results reported in Figs. 1, 2 and 3, the 'concentration parameter' of the acyl chains in the membrane may be estimated: 50% of 0.5 nmol of acyl-CoAs are bound to a volume of membrane which cannot exceed 200 nl for an assay using 10–20 μg

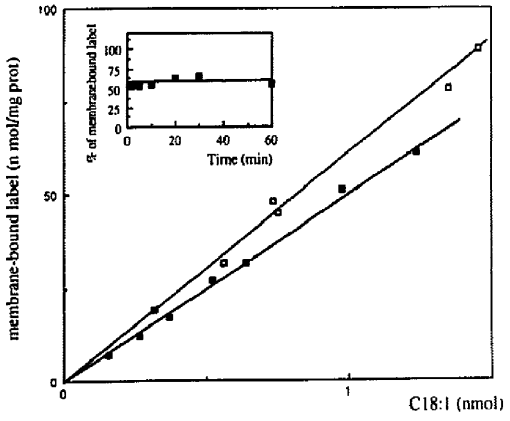


Fig. 3. Binding of the oleoyl-CoA to native or heat-treated microsomes. Various amounts of labeled oleoyl-CoA were incubated for 30 min at 37 °C with 10 μg of native (□) or heat-inactivated (■) microsomes from mouse sciatic nerves, in 0.05 M Tris-HCl buffer (pH 7.5) The membrane bound radioactivity was determined as described in Experimental procedures. (Inset) To 10 μg microsomal proteins, 0.5 nmol of [1 – ^{14}C]oleoyl-CoA (52 Ci/mol) were added for the various times indicated, at 37 °C, in 50 μl 0.05 M Tris-HCl buffer (pH 7.5) The membrane bound radioactivity was determined as described in Experimental procedures.

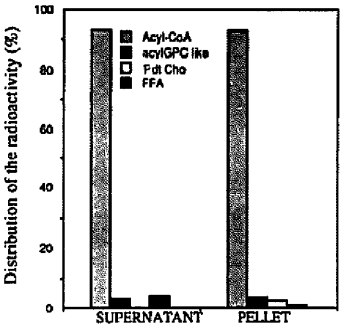


Fig. 4. Acyl-CoA metabolism by heat-inactivated microsomes from mouse sciatic nerves. To 10 μg microsomal heated proteins, 0.5 nmol of [1 – ^{14}C]oleoyl-CoA (52 Ci/mol) were added for 30 min, at 37 °C, in 50 μl 0.05 M Tris-HCl buffer (pH 7.5). The lipids were analyzed as described in Experimental procedures. PdtCho means lipid migrating as PdtCho on HPTLC plates. The total label (membrane bound and in the aqueous phase) of acyl-CoA, FFA, acyl-GPC and PtdCho-like are expressed as the percentage of total radioactivity administered.

membrane proteins. This shows that the 'concentration' of the substrate within the membrane may reach at least 1.2 mM even if the initial concentration in the aqueous phase is only 10 μM. Consequently, in agreement with our previous theoretical analysis [8] the first important role of the membrane is to concentrate the substrate. In the case of the microsomes under study, the concentration in the membrane will systematically be higher by at least two orders of magnitude than in the aqueous phase. Accordingly, the significance of a parameter such as the apparent K_M will have to be analysed according to the local concentration of amphiphilic substrates, partition coefficient..., as discussed for example in Bessoule et al. [8].

Metabolism of the acyl-CoAs

As seen in fig. 3, the distribution of the radioactivity from [1 – ^{14}C]oleoyl-CoA between microsomes and the aqueous phase is not greatly changed with heat-treated membranes. But this apparent identical partition masks two dramatically different situations.

In the case of heat-inactivated microsomes, the label of the membranes or of the aqueous phase (after 30 min of incubation) is chiefly ($\geq$ 90%) found in the acyl-CoA fraction (Fig. 4), thus the inactivated microsomes allow a measurement of the partition without interference due to any metabolism.

On the other hand, with native microsomes, there is a high level of acyl-CoA hydrolysis as shown by studying the metabolism of 0.5 nmol of acyl-CoA by sciatic nerve microsomes as a function of time. As shown in Fig. 5, there is a continuous decrease of the label in the acyl-CoA fraction; more than 15% are metabolized after 2 min, about 50% after 10 min and more than 60% after 30 min. The decrease of the label in the acyl-CoAs is conconitent with an increase of the label in the free fatty acids which accounts for 10% of the total radioactivity after 2 min, 30% after 10 min and almost 50% after 60 min. Some transacylation seems also to have occurred though no acyl acceptor was added to the reaction mixture: 1.5% of the initial label were found in a lipid migrating as PtdCho after 2 min, about 10% after 10 min and a plateau was reached

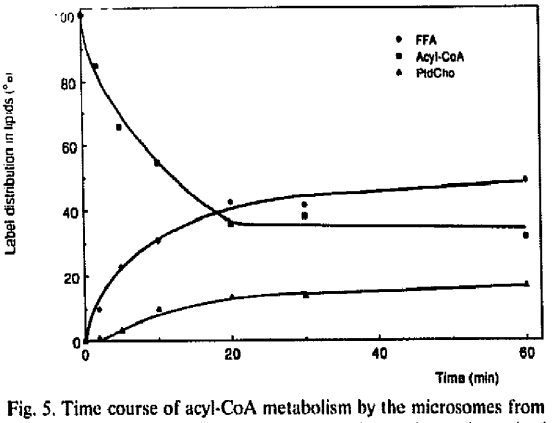


Fig. 5. Time course of acyl-CoA metabolism by the microsomes from mouse sciatic nerves. To 10 μg microsomal proteins, 0.5 nmol of [1 – ^{14}C]oleoyl-CoA (52 Ci/mol) were added for the various times indicated in the figure, at 37 °C, in 50 μl 0.05 M Tris-HCl buffer (pH 7.5) The lipids were analyzed as described in Experimental procedures. PdtCho means lipid migrating as PdtCho on HPTLC plates. For each time, the total label (membrane bound and in the aqueous phase) of acyl-CoA, FFA and PtdCho are expressed as the percentage of total radioactivity administered.

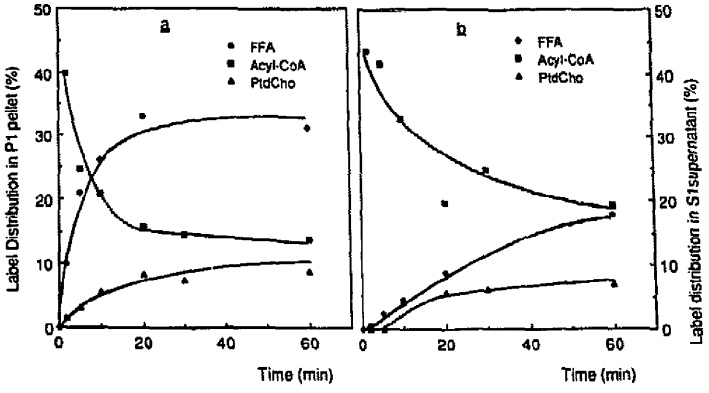


Fig. 6. Time course of the label distribution into the microsomes (P1) and the aqueous phase (S1). To 10 μg microsomal proteins, 0.5 nmol of [1 – ^{14}C]oleoyl-CoA (52 Ci/mol) were added for the various times indicated, in 50 μl 0.05 M Tris-HCl buffer (pH 7.5) The P1 pellet and S1 supernatant were prepared and the lipids were analyzed as described in Experimental procedures. The results are expressed as the percentage of the label administered. PdtCho means lipid migrating as PdtCho on HPTLC plates.

after 20 min incubation when this lipid accounted for 15% of the total radioactivity.

But, the main information to be gained from these experiments is that even if the metabolism of the acyl-CoAs by native microsomes from mouse sciatic nerves is high, the label bound to the microsomes remains constant as a function of time and almost equal to the label bound to heat-inactivated membranes (Fig. 3).

Effect of the addition of CoASH on the partition of oleoyl-CoA

When the binding of oleoyl-CoA to the microsomes (10.5 μg) was studied in the presence of exogenous CoA (0.5 nmol) there was a slight decrease of the radioactivity found in the membranes from 58.3 ± 1.5% (three assays) to about 46.5% (two assays) of acyl-CoA administered. When CoA (0.5 nmol) was preincubated for 20 min. with the microsomes prior to the addition of oleoyl-CoA, the binding dropped from 58.3 ± 1.5% (control, three assays) to 37 ± 3.3% (four assays) of acyl-CoA administered. However, the CoA-induced decrease of the binding is not high and does not exceed 36% of the maximal value observed in the absence of the CoA.

Kinetics of partition and metabolism of the acyl-CoAs

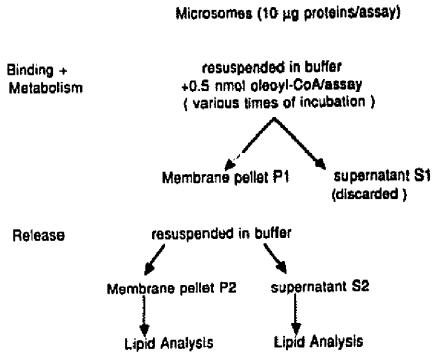
The separate analysis of the label distribution among the lipids of the membrane and aqueous compartments was carried out and the results are given in the Figs. 6a and 6b. During the FFA appearance, there is a parallel decrease of the label of the acyl-CoAs in the membranes and in the aqueous phase.

The metabolism occuring in the membrane is clearly illustrated by the appearance of free fatty acids which are rapidly formed in the membrane since after 2 min all the label of free fatty acids is almost exclusively membrane-bound. The label of the fatty acids is always higher in the membranes than in the aqueous phase though the difference tends to diminish.

Thus, it is easy to calculate that the amount of acyl-CoA which has been bound to the membrane increases as a function of time (at any given time, this amount of acyl-CoA may be assimilated to the sum of the radioactivity actually observed in the membrane acyl-CoAs, free fatty acids (whatever their location) and, in the case of acylation, phospholipids). It is clear that the decrease in the aqueous acyl-CoAs (Fig. 6) is exactly accounted for by an increase in the amount of acyl-CoA which has been bound to the membrane which may reach 80% of the total label after 60 min. These results strongly suggest that though the total label of the membrane does not vary as a function of time, there is a continuous transfer of aqueous acyl-CoAs to the membrane, a continuous hydrolysis of the membrane-bound oleoyl-CoA and a continuous release of the metabolites (FFA) from the membrane. In order to gain more information concerning the release of membrane-bound amphiphilic molecules, further experiments were designed.

Release of labeled molecules from the membrane to the aqueous phase

The rationale of the assay is described in the Scheme I. The label of S2 and P2 is supposed to reflect the partition of the radioactive membrane-bound material between the membrane and the aqueous phase. Using 0.42 ± 0.1 nmol oleoyl-CoA bound to 10 μg microsomal proteins, we observed 0.101 ± 0.012 nmol (25.6 ± 7%) in S2 (n = 10). This percent of release was not greatly influenced by the amount of acyl-CoA bound to P1 since the label associated with P2 and S2 fractions



Scheme I. Protocol used to determine the release of molecules from the membrane. The label associated with P1, P2 and S2 was determined; the lipids from P2 and S2 were analyzed by HPTLC and their radioactivities determined as described in Experimental procedures.

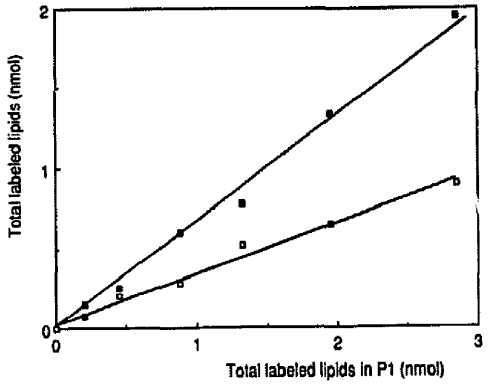


Fig. 7. Labeled lipid release from the preloaded microsomes as a function of the membrane-label. To 10 μg microsomal proteins, variable amounts of [1 – ^{14}C]oleoyl-CoA (52 Ci/mol) were added, for 30 min at 37 °C in 50 μl Tris-HCl buffer (pH 7.5) The membrane P1 pellet was prepared, homogenized and its radioactivity measured. The P2 pellet and the S2 supernatant were prepared and their radioactivities analyzed. The results are given as the radioactivity of P2 and S2 (expressed as nmol/assay) as a function of the lipid label of P1 (nmol/assay). ■, P2 pellet; □, S_2 supernatant.

are directly proportional to the label associated with P1 (Fig. 7) and the mean slopes are around 0.7 and 0.3, respectively.

Release as a function of time

Constant amounts of microsomes (10 μg proteins) were resuspended in the presence of 0.5 nmol oleoyl-CoA. After 2, 5, 10, 20, 30 and 60 min, the membrane pellet (P1) and the supernatant (S1) were prepared as described in Experimental procedures. The P1 pellet was resuspended in the same buffer and left for 2 min; then the membrane pellet (P2) and the supernatant (S2) were prepared. The lipids of both fractions were analyzed. The results are given in the Table I.

This assay allows to study the release of the acyl-CoA and their metabolites from the membrane. Whatever the time of incubation of the microsomes, there is only a slight variation of the label release from the P1 pellet and the label in P2 varies from 77% (after 2 min) to 67% (after 60 min) of the initial radioactivity of the P1 pellet. The release of the radioactivity from the P1 membrane pellet to the aqueous environment affects the acyl-CoAs, the PtdCho-like molecules and the free fatty acids but not the lipid migrating like acyl-GPC. The latter result is in good agreement with our previous indications that there is only a limited, if any, loss of membranes during the preparations of P1 and P2 pellets. The maximal release of acyl-CoA is 15% of the membrane acyl-CoA ($t = 60$ min) and except for the first assay, accounts for only $2.7 \pm 0.8\%$ of the initial radioactivity (mean value ± S.D. of five assays). This value may be compared with the total label in S2, i.e. the total release which is 27.8 + 3.9% of the total label (mean value ± S.D. of six assays). Consequently, the acyl-CoA release accounts for about 10% of the total release.

TABLE I

Time course of the distribution of the label between the microsomes (P2) and the aqueous phase (S2)

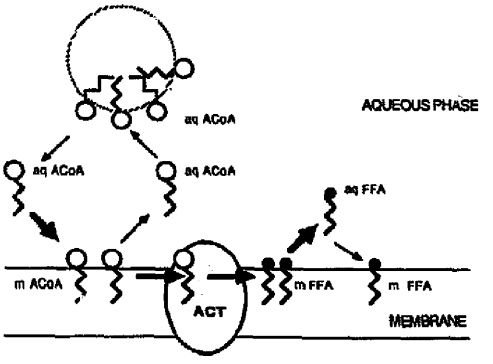
The values P2 and S2 refer to the label found in P2 and S2 after separation of the membranes (P2) and aqueous phase (S2) as described in the scheme I. To 10 μg microsomal proteins, 0.5 nmol of [1 – ^{14}C]oleoyl-CoA (52 Ci/mol) were added for the various times indicated in 50 μl 0.05 M Tris-HCl (pH 7.5). The P2 pellet and the S2 supernatant were obtained and the lipid analysis were carried out as described in Experimental procedures. The value 100% refers to the activity associated with the fraction P1. For each time under study, the sum of label in P2 and S2 is 100% (and is equal to the radioactivity of the P1 pellet).

Time (min)	Acyl-CoA			Acyl-GPC like			PtdCHO like			FFA			Total radioactivity	
	P1	P2	S2	P1	P2	S2	P1	P2	S2	P1	P2	S2	P2	S2
2	62.1	52.4	9.7	3.2	3.2	–	8.1	4.9	3.2	26.2	16.4	9.8	77	23
5	39.3	36.5	2.8	5.5	2.2	3.2	11	5.5	5.5	44.1	32	12.1	76.2	23.8
10	32.3	30.7	1.6	2.1	2.1	–	13.6	9.4	4.2	52.1	29.2	22.9	71.4	28.6
20	26.7	23.9	2.8	4.2	4.2	–	16.8	8.4	8.4	52.1	33.8	18.3	70.5	29.5
30	20.8	18.5	2.3	1.7	1.7	–	19.1	13.9	5.2	58.3	37.5	20.8	71.6	28.3
60	24.5	20.6	3.9	2.5	2.5	–	11	5.2	5.8	62	38.8	23.2	67.1	32.9

As seen in Table I, there is an important free fatty acid release: 35% to 43% of the total free fatty acids are found in the aqueous phase, indicating that the partition of the fatty acids is slightly different from that of the acyl-CoAs in that more free fatty acids are left in the aqueous phase. On the other hand, the free fatty acids account for 40–50% of the total released label when the microsomes have been incubated for 2 min and 5 min with oleoyl-CoA and this value jumps to 60–80% for longer incubation times.

These results demonstrate that the acyl-CoAs bound to the membrane may be partly released and then subtracted from further metabolism, or remain as such within the membrane, or are metabolized giving rise to free fatty acids, among other products, which in turn are partly released in the aqueous phase. Accordingly, the Scheme II may summarize the main parameters reported in this study. The aqueous acyl-CoA (aq ACoA) partitions between membrane and water. The membrane-bound acyl-CoA (m ACoA) is partly released in the aqueous phase, partly metabolized within the membrane. The membrane free fatty acids (m FFA) remain partly membrane bound and are partly released in the aqueous phase (aq FFA). The aqueous free fatty acids, in turn, could partly partition with the membrane (m FFA). This scheme explains that there is a net movement of the aqueous acyl-CoA towards the membrane, and a net movement of the free fatty acids from the membrane to the aqueous phase, so that the label in the membrane and in the aqueous phase remains nearly constant, whereas its distribution between lipids varies as a function of time firstly in the membranes and then in the aqueous phase.

We have then investigated the effect of the addition of BSA (i.e., a protein able to bind both acyl-CoAs and free fatty acids) on the partition and the metabolism of acyl-CoAs.



ACT : Acyl-CoA thioesterase

Scheme II. The binding of acyl-CoA to the membrane allows its metabolism. The metabolites (in this case FFA) may be released from the membrane. The partition coefficient in terms of distribution of the acyl moieties does not vary.

Modification of the partition of acyl-CoA by BSA

In our hands, according to the amount of BSA added to the experimental medium, $10 \pm 2.6\%$ (five assays) of 0.5 nmol oleoyl-CoA were bound to 10 μg microsomal proteins in the presence of 50 μg BSA (or 20 to 25% when this amount was lowered to 20 μg), instead of $57.7 \pm 5.3\%$ in its absence. The consequence is, as already reported [10], a reduction of the effective (i.e., membrane bound) substrate concentration and, in this way, a decrease of the apparent affinity. Thus, the first effect of the BSA is to compete with the membrane for the binding of the acyl-CoA. This effect has

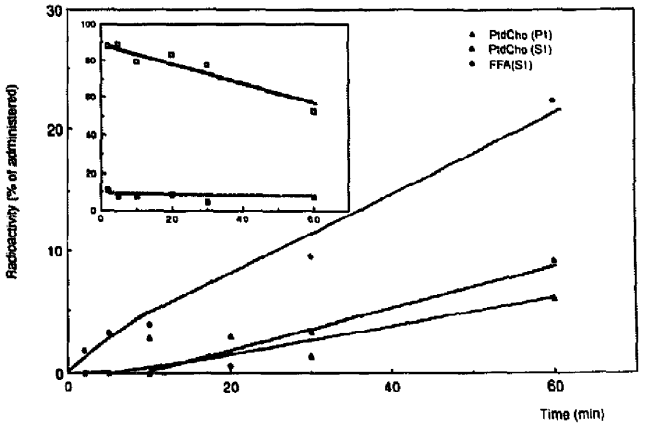


Fig. 8. Time course of binding and metabolism of [1-14C]oleoyl-CoA in the presence of BSA. Same experimental conditions as in Fig. 5, but in the presence of 50 μg BSA in each assay. For each incubation time, the label of the PtdCho and FFA either in P1 or in S1 are expressed as the % of the initial administered label. PdtCho means lipid migrating as PdtCho on HPTLC plates. (Inset) Acyl-CoA label in Pi (■) and in S1 (□) as a function of time in the presence of 50 μg BSA. Results are given as the % of the label administered.

been demonstrated previously and it has been shown that albumin has 5–6 high affinity sites which bind strongly the acyl-CoA and that the membrane competes favorably only when the high affinity sites of the albumin are saturated [10].

As a function of time, there is only a slight variation of the distribution of the label in the membranes (PI pellet, $10 \pm 2.7\%$) and in the aqueous phase. This result suggests that the partition of the acyl-CoA between the membrane and the aqueous phase (i.e., the binding to the BSA) is a fast phenomenon as compared to the time scale used in this study.

The analysis of the distribution of the label between P1 and S1 was also carried out. The results are given in the Fig. 8. Radioactivity is associated with four lipid species: acyl-CoAs, free fatty acids, the acyl-GPC-like and PtdCho like molecules. As already observed in the absence of BSA, there is an acyl insertion into a lipid migrating as PtdCho in the absence of any exogenously added acyl acceptor. This acylation remains weak (< 5% of administered acyl-CoA) for 30 min but reaches approx. 15% for a 60 min incubation. However, the main difference with the results obtained in the absence of BSA is the higher percentage of unmetabolized acyl-CoA whatever the time of incubation (98% after a 2 min incubation and approx. 60% after 60 min). This is of course related with a sharp decrease of the formation of labeled free fatty acids which reach 22% of the total substrate in 60 min and less than 10% in 30 min. This well-known effect of the BSA on the activity of the acyl-CoA thioesterase activity is probably due to the decrease of the acyl-CoA binding to the microsomes, which, as seen in Fig. 8 (inset) is always < 10% of administered, whatever the time of incubation. Thus, the formation of free fatty acids from the membrane bound acyl-CoAs is reduced, though it represents after 60 min more radioactivity than that of the membrane bound acyl-CoAs; this observation suggests that, even in the presence of BSA, there is a net flux of acyl-CoAs from the aqueous phase to the membrane and that this flux is related to the metabolism within the membrane. In addition, the free fatty acids are strictly associated with the aqueous phase, no label was found in the microsomes in a 0–60 min range, suggesting again that there is a net flux of the free fatty acids from the membrane to the aqueous phase. The existence of a transfer of fatty acids between phospholipids vesicles and albumin has been clearly demonstrated earlier [4,5]. Hamilton and Cistola [5] have established that the transfer is pH-dependent and that in a system containing equal amounts of BSA and phospholipids and 4 mol of oleic acid per mol of BSA, most (> 90%) of the oleic acid was bound to the BSA at pH 7.3, whereas the reverse was observed at pH 5.4. This reversible process could be due to the differences in the pK_a of the fatty acids bound to the BSA ($pK_a = 4.2$) or to phospholipids ($pK_a = 7.6$). It was proposed that a slightly alkaline pH could provide the driving force for the removal of the fatty acids from the phospholipid vesicles and their binding to the BSA. In our hands, the high BSA to membrane protein ratio (approx. 5) and the midly alkaline pH (7.4) are consistent with a constant and total release of the free fatty acids from the membranes as soon as they are formed by the membrane acyl-CoA thioesterase. The implication of the absence of membrane bound free fatty acids is that the removal of the fatty acids from the membrane is a faster phenomenon than their formation. This is again in close agreement with the evaluation made by Hamilton and Cistola [5] who suggested a fatty acid exchange rate between PtdCho vesicles and BSA on the time-scale of seconds or minutes.

Discussion

Numerous methods may be chosen to determine the partition coefficient of amphiphilic molecules; for example hygroscopic desorption, centrifugation and fluorescence titration, which according to Pjura et al. [16] led to similar results. In the case of a subsequent study of the metabolism of the membrane bound amphiphilic substrates, the choice of the centrifugation technique, though time-consuming, is highly recommended for at least three reasons: (i) It is the only one allowing to discriminate physically the membrane bound and the free (as monomers or eventually micelles) acyl-CoAs, i.e., in this case, the metabolizable or the non-metabolizable molecules. (ii) It allows to study the release of the amphiphilic molecules from the membranes. (iii) It allows to study the fate of the membrane bound substrate without taking into account the unbound substrate. The results reported here are in good agreement with those of Pjura et al. [16], for the usefulness of this technique. Conrad and Singer [17] raised the possibility that there are interactions of acyl-CoA micelles bound to the membranes.The micelles bound artefactually to the membranes would be undistinguishable from the true binding of acyl-CoAs with membrane. In our hands, the acyl-CoA concentration was usually 10 μM or less, in the absence of any binding, i.e., probably far below the CMC which was recently reported to be above 30 μM [1], largely higher than the previously reported values of 3–4 μM [18]. We have studied the potential losses of membranes during the microultracentrifugation. Probably because of the conditions of sedimentation, we did not observe a high loss of proteins and even in the case of low amounts of proteins (5 to 10 μg),the maximal loss was $12.6 \pm 1.7\%$ (7 assays), probably because of the parameters of ultracentrifugation (short time: 10 min, high speed: $184\,000 \times g$).

Under the experimental conditions used by Pjura et

al. [16], however, as much as 40% of the total plasma membrane seemed to remain in the supernatant following centrifugation at 100 000 × g for 30 min. This is not the case in our hands and we did not detect a significant presence of membrane proteins in the supernatant under the experimental conditions used throughout this study. This confirms as hypothesized from the binding curves, that in the presence of the amounts of acyl-CoA we used, there is no measurable solubilization of the membrane via a detergent effect of these molecules.

The interaction of oleoyl-CoA with phospholipid bilayers has already been studied [19]. Using spin labeled acyl-CoA, these authors have demonstrated that when a given amount of acyl-CoA ($5 \cdot 10^{-5}$ M) was mixed with mitochondrial phospholipids ($5 \cdot 10^{-4}$ M), the ratio of membrane-bound acyl-CoA to the aqueous acyl-CoA may vary between 5 and 40, depending whether the oxazolidine group is located near the carboxyl or the methyl end of the acyl moiety of the acyl-CoA. This study has been developed [1]. It was shown that the acyl-CoA binds to PtdCho vesicles and as a consequence increases the density of the vesicles which may be separated from the oleoyl-CoA-free vesicles by centrifugation on sucrose gradients. The addition of cholesterol to the vesicles decreases the binding of the oleoyl-CoA as well as the addition in the aqueous phase of BSA (in a molar ratio of 1); in the first case the decrease of the binding is probably due to an increase of the surface density as shown by De Young and Dill [2]; and in the second case, the decrease of the binding results from the interaction of the oleoyl-CoA with the albumin which is known to bind equimolecular amounts of oleoyl-CoA [20,21].

Expression of the binding and of the release of acyl-CoAs as a function of acyl-CoA and of microsomes amounts

The partition of amphiphilic substrates between water and biological membrane has been questionned and according to De Young and Dill [2], this partitioning could be of a fundamentally different nature than partitioning into bulk oil or octanol phases; accordingly, the surface density of the biological membrane is expected to affect the partition coefficient, which, as a consequence should be variable [2]. The results presented in this paper show that under the conditions used:

(a) The binding of increasing amounts of acyl-CoA to a constant amount of membrane (expressed as membrane proteins) is a direct function of the amount of acyl-CoA present in the assay. Hence:

$$(\partial M_1 / \partial S_O)_P = \text{constant} \qquad (1)$$

where M_1 is the amount of labeled molecules bound to the membrane and S_O is the total amount (bound and unbound) of the substrate.

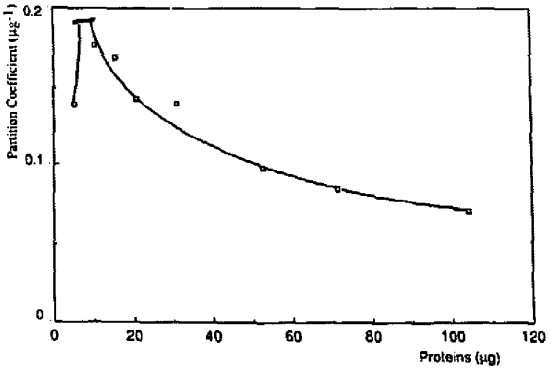


Fig. 9. Variation of the partition coefficient as a function of the amount of membrane proteins. The partition coefficient is calculated from the results reported in Fig. 1 and assumed to be equal to: $(M_1/P)/(S_O - M_1)$.

(b) The binding of a constant amount ($S_O = 0.5$ nmol) of substrate as a function of the membrane protein amount P ($\geqslant 5$ µg) is hyperbolic (Fig. 1) and experimentally it can be described by the equation:

$$M_1 = (b - (a/P))S_O \qquad (2)$$

From Fig. 1 it can be calculated that $a = 2.5$ µg and $b = 0.88$. The experimental value of b is 0.88 and not 1 probably because 12% of membrane were lost during the experimental procedure.

The Eqn. 2 shows that the binding of the substrate is not directly proportional to S_O: the term $-(a/P)$ is a 'release factor' which will contribute largely to the value of M_1 when the amount of proteins (membrane) is low; in other words, it explains that (and why) the release will increase when there is 'overloading' of acyl-CoA bound to the membrane. Hence the partition coefficient of acyl-CoA is not constant for a low amount of biological membrane, as illustrated in Fig. 9. On the other hand, when P increases there is a decrease of the value of (a/P) which tends to be negligible and consequently the partition coefficient tends to be constant.

The release of labeled molecules from membranes into a buffer devoid of acyl-CoAs has also been analyzed. It may be calculated that the amount of labeled molecules released (S_2) from a membrane can be described by the equation:

$$S_2 = (b' + (a'/P))M_1 \qquad (3)$$

The experimental values of a' and b' were: $a' = 2.7$ µg and $b' = 0.03$. It can be noticed that: $a \approx a'$; $b \approx 1$ and $b' \approx 0$. These results strongly suggest that the partition of the labeled molecules depends on the amount of membrane but (as expected) does not depend on their initial location (membranous or aqueous phase).

Consequences upon the analysis of the enzyme activities

Since the substrate is strictly membrane-bound, it is more advisable to consider the density of the substrate into the membrane rather than its 'global concentration' in the incubation mixture to analyse the enzyme activity (see also Ref. 8). From Eqn. 2, the acyl-CoA density in the vesicles may be estimated:

$$d = M_1/P = S_O(bP - a)/P^2 \quad (4)$$

Hence, it is easy to calculate $(\partial d/\partial P)_{S_0}$, and to show that d has a maximal value when $P = 2a/b$; i.e., when $M_1 = S_O \cdot b/2$. Under our experimental conditions, d reaches its maximal value when $P \approx 5\ \mu g$.

Cassagne et al. [13] showed recently that the total activity of the acyl-CoA:acyl-GPC transacylase from microsomes of Trembler mouse sciatic nerves tends towards a plateau and that the specific activity decreases when the protein amount increases from 5 to 40 μg. This can be due to the fact that d decreases as a function of P: calculating $d((0.88\ P - 2.5\ )/P^2$ this study) and the specific activity from the data of Cassagne et al. [13], we show that the variation of 1/specific activity as a function of $1/d$ is linear. Thus, the activity of the acyl-CoA:acyl-GPC transacylase could be Michaelian and the decrease of the specific activity when the protein amount increases simply reflects a 'dilution' of the substrate within the membrane. These results and the analysis of the results presented here are in close agreement with those reported earlier by Noy and Zakim [9] concerning an acyl-CoA ligase. The theoretical study already undertaken [8] and developed here, emphasizes the prominent role of the local concentration of the substrate near the membrane enzymes, irrespective of the bulk concentration.

Analysis of the partition coefficients when acyl-CoA metabolism occurs

(i) In the absence of acyl-CoA metabolism ($t = 0$ or heat inactivated microsomes), the fate of the acyl-CoAs is:

$$\text{aqACoA} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{mACoA}$$

and the partition coefficient $\Pi_1 = (\text{mACoA}/\text{aqACoA})$ inactivated microsomes $= k_1/k_{-1}$.

The fate of the acyl-CoAs by actively metabolizing microsomes is:

$$\text{aqACoA} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{mACoA} \xrightarrow{k_2} \text{FFA}$$

We have shown (Figs. 6a, 6b) that for $t \geq 10$ min, mACoA has reached a steady state, so that Π_2, the partition coefficient is $\Pi_2 = \text{mACoA}/\text{aqACoA} = k_1/(k_{-1} + k_2)$.

It appears clearly that $\Pi_1 > \Pi_2$ and therefore that the percentage of membrane-bound acyl CoA was lower with actively microsomes metabolizing than with heat-inactivated microsomes.This explain why using heat-inactivated microsomes, 51% of the acyl-CoA were membrane-bound (Fig. 3), whereas as it is shown Figs. 6a, 6b, during the hydrolysis by native microsomes, only around 40% (for $t \geq 10$ min) of the total acyl-CoA remain associated with the membrane. Between 0 and 10 min the percentage of membrane-bound acyl-CoA decreased because the system evolves from the steady state 1 to the steady state 2.

(ii) In contrast with the percentage of the membrane-bound acyl-CoA, the percentage of membrane-bound FFA varied as a function of time: 94% of FFA were bound at 2 min, 84% at 20 min and 64% at 60 min (Figs. 6a, 6b). These results may be understood if one assumes that there is a constant partition of the total aliphatic chains irrespective of their form (acyl-CoA or free fatty acids). This assumption takes into account (and explains) the fact that the distribution of the radioactivity between the membrane and the aqueous phase is constant as a function of time (Fig. 3 inset).

Assuming a constant partition of the amphiphilic aliphatic chains:

$$(\text{mACoA} + \text{mFFA})/(\text{aqACoA} + \text{aqFFA}) = \Pi_1 = k_1/k_{-1} \quad (5)$$

then:

$$\text{mFFA} - \Pi_1 \cdot \text{aqFFA} = \Pi_1 \cdot \text{aqACoA} - \text{mACoA}$$

$$= \text{mACoA} \cdot ((\Pi_1/\Pi_2) - 1) \quad (6)$$

So being the initial amount of ACoA:

$$\text{mACoA} + \text{aqACoA} = \text{mACoA} \cdot (1 + (1/\Pi_2)) = S_O - \text{aqFFA} - \text{mFFA} \quad (7)$$

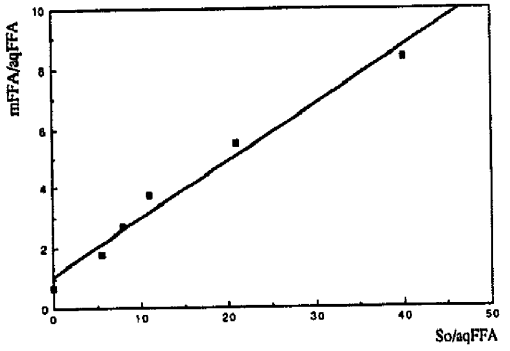


Fig. 10. Variation of the partition coefficient of FFA as a function of metabolism. The partition coefficient is calculated from the results reported in Figs. 6a and 6b.

From Eqns. 6 and 7:

$$\text{mFFA/aqFFA} = \frac{(\Pi_1 - \Pi_2)\cdot S_O}{(\Pi_1 + 1)\cdot \text{aqFFA}} + \Pi_2 \qquad (8)$$

Since aqFFA increased as a function of time (Fig. 6), mFFA/aqFFA (and therefore the percentage of membrane-bound free fatty acids) decreased.

From the Fig. 3 it can be calculated that $\Pi_1 = 1.06$ (51.4% of the total radioactivity are found in the membrane of heat inactivated microsomes) and from Figs. 6a and 6b that $\Pi_2 = 0.67 \pm 0.09$. Therefore, if the assumption is valid, mFFA/aqFFA should be linear as S_O/aqFFA and the slope must be 0.1893. From Fig. 10 we observed experimentally a linear variation and the slope (determined by computer analysis) is 0.1928. Hence, it is likely that there is a constant partition of the total aliphatic chains irrespective of their form (acyl-CoA or free fatty acids).

Conclusion

The analysis of the binding and of the release of the acyl-CoAs discussed above is sound as long as the measurement of the radioactivity is roughly an estimation of the acyl-CoAs, i.e., as long as the overall metabolism of this molecule is reasonably low. In this case which is effectively observed when the total metabolism of the acyl-CoAs is lower than 10%, several methods such as those described already in the literature may be used in conjunction with the microultracentrifugation. But if the level of metabolism increases, as shown in this study for the higher times of incubation, the hydrolysis of the acyl-CoA by the membrane should no longer be neglected. In this case, modifications of the partition of the acyl-CoAs may be observed experimentally and predicted theoretically according to the analysis developed above. Thus, in all the studies dealing with the partition of amphiphilic substrates in biological membranes, we highly recommend a complete analysis of the metabolism of these substrates and a theoretical approach of the partition function such as reported here.

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