Interaction between Spin-Labeled Acyl-Coenzyme A and the Mitochondrial Adenosine Diphosphate Carrier†

Philippe F. Devaux,* Alain Bienvenüe, Guy Lauquin, Alain D. Brisson, Paulette M. Vignais, and Pierre V. Vignais

ABSTRACT: (1) Spin-labeled long-chain (*m,n*)acyl-CoA's (general formula: $CH_3(CH_2)_mCR(CH_2)_nCOSCoA$, where R is an oxazolidine ring containing a nitroxide) inhibit anion transports through the inner mitochondrial membrane at low concentrations as ordinary long-chain acyl-CoA's do. The inhibition constant relative to the inhibition of the ADP transport in heart mitochondria by spin-labeled palmityl-CoA and stearyl-CoA is of the order of 10^{-7} M, a value which is similar to that found for natural long-chain acyl-CoA's. A short-chain spin-labeled acyl-CoA (C_5) showed no inhibitory effect in the range of concentrations tested (up to 30 μM). (2) (10,3)Acyl-CoA added to heart mitochondria at low concentrations exhibits spectra corresponding to an immobilized probe. The corresponding free fatty acid shows a higher freedom of motion between 0 and 30°. The same differences in spectra of spin-labeled acyl-CoA and spin-labeled free fatty acid were found in inner membrane vesicles from rat liver mitochondria, but not in outer membrane preparations. (3) The selective interaction of spin-labeled acyl-CoA with the ADP carrier is indicated by the release of this interaction by specific ligands of the ADP carrier, such as ADP or ATP, carboxyatractyloside, and bongkrekic acid. ADP (or ATP) and carboxyatractyloside rendered the spin-labeled (10,3)acyl-CoA nearly as mobile as the (10,3) free fatty acid. No effect was obtained with AMP, GDP, or UDP which are not transported by the ADP carrier. Bongkrekic acid, another specific inhibitor of the ADP carrier, was inactive when added alone; however, it was effective when added together with amounts of ADP which are ineffective *per se*. (4) The electron spin resonance (esr) spectrum observed at low concentrations of (10,3)acyl-CoA arises from (10,3)acyl-CoA bound to the ADP carrier. At higher concentrations the (10,3)-acyl-CoA is more mobile, suggesting that the bulk of the label is also present in the lipid phase of the membrane. Spin-labeled acyl-CoA's incorporated into a sonicated dispersion of lipids extracted from heart mitochondria exhibited similar mobile spectra. (5) When the oxazolidine ring is moved down the hydrocarbon chain of the acyl-CoA, the binding features tended to disappear. Whereas nitroxide–protein interactions could be easily measured with the (10,3)acyl-CoA and the (7,6)acyl-CoA, much less or even no significant interactions could be detected with the (5,10)acyl-CoA or the (1,14)acyl-CoA. (6) The above results suggest that spin-labeled long-chain acyl-CoA added to mitochondria binds by its polar moiety to the ADP carrier. The acyl chain interacts with the ADP carrier protein over a length of 10–15 Å. The remaining portion of the acyl chain experiences a fluid lipid environment.

Spin labels have been shown to be useful tools for the determination of the fluidity and diffusion of phospholipids in biological membranes (for a review see McConnell and McFarland, 1970; Jost *et al.*, 1971; Keith *et al.*, 1973). The topology and the dynamics of proteins of cellular membranes are a matter of current interest. Among these proteins, those endowed with specific transport functions are particularly interesting. The size and motion of carrier proteins can be evaluated by means of spin labeling if the following requirements are fulfilled. (1) Spin-labeled substrate or inhibitor analogs must retain the biological and chemical properties of the unlabeled molecules measured by binding affinity, binding capacity, and ability to be transported or to inhibit. (2) The spin-labeled substrates or inhibitors which bind selectively to the carrier should not undergo significant metabolic degradation. (3) The affinity of the probe for its binding site and the number of sites have to be high enough for the spin-labeled complex to be clearly distinguished from the free spin label by electron spin resonance (esr) measurements.

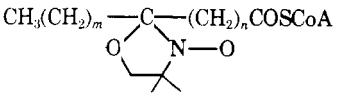
Long-chain acyl-CoA's inhibit the functioning of a number of anion carriers in the inner mitochondrial membrane (for a review, see Morel *et al.*, 1974). The ADP transport is by far the most sensitive to the inhibitory effect of long-chain acyl-CoA's (Pande and Blanchaer, 1971), the inhibitor constant for the ADP transport (K_i^{ADP}) being one to two orders of magnitude lower than the inhibitor constant related to the transport of citrate, malate, or phosphate (Morel *et al.*, 1974). Another protein of the inner mitochondrial membrane very sensitive to long-chain acyl-CoA is the pyridine-nucleotide transhydrogenase (Rydström, 1972). However, the transhydrogenase in intact mitochondria does not react readily with long-chain acyl-CoA (see Results).

Fatty acids labeled in their hydrocarbon chain by a nitroxide radical can be converted to spin-labeled fatty acyl-CoA derivatives to probe the microenvironment and the size of the ADP carrier in the inner mitochondrial membrane. This paper describes the preparation of spin-labeled acyl-CoA's and their interaction with the ADP carrier. The specificity of this interaction is studied using ligands specific for the ADP carrier.

† From Laboratoire de Biophysique Moléculaire, G.P.S.-E.N.S. Tour 23, Université Paris VII (P.D., A.B., and A.D.B.), 75221 Paris Cedex 05, France, and Laboratoire de Biochimie, Département de Recherche Fondamentale du Centre d'Etudes Nucléaires et Université Scientifique et Médicale de Grenoble, France (G.L., P.M.V., and P.V.V.). *Received July 16, 1974.* This investigation was supported by research grants from the "Délégation Générale à la Recherche Scientifique et Technique" and the "Centre National de la Recherche Scientifique" (E.R.A. No. 36).

Experimental Section

Synthesis of Spin-Labeled Acyl-CoA. Spin-labeled acyl-CoA's having the following general structure



(*m*, *n*) acyl-CoA

were prepared from the anhydrides of spin-labeled fatty acids by a modification of the method of Vignais and Zabin (1958).

The spin-labeled palmitic and stearic acids (10,3)FA, (7,6)FA, and (1,14)FA were synthesized according to the method of Hubbell and McConnell (1971); (5,10)FA and (12,3)FA were Synvar products (Synvar Associates, Palo Alto, Calif.). The corresponding anhydrides were prepared by treating 0.1 mmol of (*m*,*n*)FA[1] with 0.08 mmol of dicyclohexylcarbodiimide in 1 ml of carbon tetrachloride (Sellinger and Lapidot, 1966). The precipitated dicyclohexylurea was removed by filtration through sintered glass and the filtrate checked by infrared (ir) spectroscopy (the anhydride yield averaged 80–90%). To prepare the acyl-CoA derivatives carbon tetrachloride was removed under reduced pressure and replaced by peroxide-free dried tetrahydrofuran (H_4furan) (1 ml). Then 0.15 ml of this solution of spin-labeled anhydride was added to 1 ml of peroxide-free H_4furan together with CoA dissolved in water. The CoA solution was made as follows: 10 mg of CoA and 1.5 mg of EDTA were dissolved at 0° in 1 ml of water and the pH was adjusted to 7.5–8.0 with sodium bicarbonate. The mixture of CoA and spin-labeled fatty acid anhydride in aqueous H_4furan was stirred by argon bubbling. It was sometimes necessary to add a few drops of water or H_4furan to achieve a homogeneous mixture. The esterification was allowed to proceed for 10–15 min at 0°.

An aliquot of the reaction mixture was tested for -SH with nitroprusside reagent. Anhydride was added until the -SH test became negative, indicating that all of the CoA had reacted. The presence of a thio ester was demonstrated by reappearance of a positive -SH test after hydrolysis by 1 N NaOH. The pH of the mixture was adjusted to 6 with 0.1 N HCl and the remaining spin-labeled fatty acid was extracted with four 4-ml fractions of ethyl ether. The powder obtained by freeze drying was dissolved in 0.5 ml of methanol for thin-layer chromatography on cellulose (Eastman Kodak) with butanol–acetic acid–water (5:2:3, v/v) as eluent (Pullman, 1973). The ultraviolet (uv) absorbing band moving with the same R_F (0.7) as authentic unlabeled palmityl-CoA was scraped off the plate and washed with methanol and the product dried under vacuum. The overall yield of spin-labeled acyl-CoA was approximately 50%, based on the amount of CoA added. The concentration of (*m*,*n*)acyl-CoA in water was estimated by uv absorption assuming an extinction coefficient of 16,000 at 260 nm (Weidman *et al.*, 1973). The radical concentration was determined by esr spectroscopy by comparison of the amplitudes of the mid-field lines of (*m*,*n*)acyl-CoA and (*m*,*n*)FA of known concentration in water. This latter determination gave 70–80% of the CoA concentration determined spectrophotometrically. Spin-labeled acyl-CoA's were stored in water at −18°.

[1] Abbreviations used are: (*m*,*n*)FA, spin-labeled fatty acid; (*m*,*n*)acyl-CoA, spin-labeled acyl-coenzyme A; these are according to the formula given in the Experimental Section; H_4furan, tetrahydrofuran.

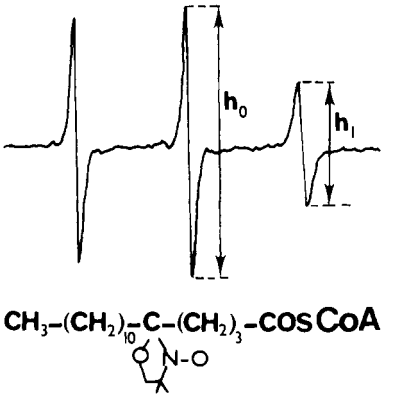


FIGURE 1: Esr spectrum of (10,3)acyl-CoA in water. The ratio h_0/h_1 can be used to determine the tumbling rate experienced by the probe (see, for instance, McConnell and McFarland, 1970). This in turn depends on the position of the probe on the molecule. When the *N*-oxyloxazolidine is moved down the hydrocarbon chain toward the methyl terminal and away from the bulky CoA moiety, the ratio h_0/h_1 changes. For the (10,3)acyl-CoA, (7,6)acyl-CoA, (5,10)acyl-CoA, and (1,14)acyl-CoA, one finds respectively (at room temperature and in water): h_0/h_1 = 2.30, 2.20, 1.98, and 1.45.

The shape of the esr spectrum of a long-chain spin-labeled acyl-CoA in water depends on the position of the probe on the molecule. Indeed when the oxazolidine group is close to the CoA moiety ((10,3)acyl-CoA) the tumbling rate experienced by the nitroxide is lower than when the probe is at the end of the alkyl chain ((1,14)acyl-CoA) (see Figure 1).

A short-chain acyl-CoA was prepared from a five carbon atom fatty acid, (0,2)FA. (0,2)FA was synthesized from levulinic acid using the technique of Keana *et al.* (1967) with slight modifications. The levulinic acid was first esterified with 1-butanol in the presence of catalytic amounts of HCl. The formation of water-insoluble butyl ester allowed the easy removal of excess 2-amino-2-methyl-1-propanol used. The (0,2)acyl-CoA prepared from (0,2)FA anhydride as described above migrated on a thin layer of cellulose in butanol–acetic acid–water (5:2:3, v/v) with an R_F of 0.45.

Esr Measurements. The esr spectra were recorded using either a Thomson CSF, TSN 254-2, X-band spectrometer or a Varian V4502. Samples in 50-μl glass capillaries or in a 100-μl flat quartz cell were mounted in a variable temperature accessory and the temperature controlled within 1°.

Spin-labeled fatty acids and spin-labeled acyl-CoA's were incorporated in the mitochondrial membrane by simply adding the spin label dissolved in water to a suspension of mitochondria in isotonic sucrose. At or above room temperature the esr signal of the spin label incorporated in the membranes of rat mitochondria decreased slowly due to reduction. To overcome this difficulty, in most experiments with rat heart mitochondria the tissue preparation was preincubated for 0.5–1 hr at 0° with 4–10 mM ferricyanide which corresponds to 50–125 nmol of ferricyanide per mg of protein. At these ratios of ferricyanide to protein the rate of [^{14}C]ADP translocation in rat heart mitochondria was not significantly modified.

The esr spectra of spin-labeled fatty acids in lipid bilayers and in various biological membranes have been extensively analyzed (see, for instance, Jost *et al.*, 1971; Hubbell and McConnell, 1971; Keith *et al.*, 1973). In this study both spin-labeled fatty acids and acyl-CoA's were used. We shall mainly consider the spectral differences observed in mitochondrial membranes with these two types of label. We

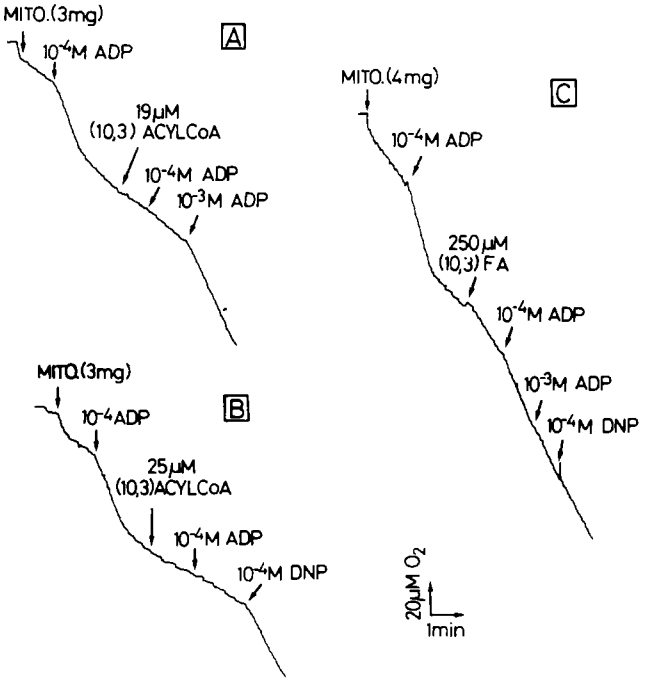


FIGURE 2: Effect of (10,3)acyl-CoA and (10,3)FA on the ADP-stimulated respiration of rat liver mitochondria: (A) the inhibition by (10,3)acyl-CoA is reversed by a high concentration of ADP; (B) the inhibition by (10,3)acyl-CoA is reversed by 2,4-dinitrophenol; (C) at 250 μM (10,3)FA is not inhibitory but uncouples. The reaction medium contained 110 mM KCl, 16 mM phosphate buffer (pH 7.4), 6 mM $MgCl_2$, and 10 mM glutamate.

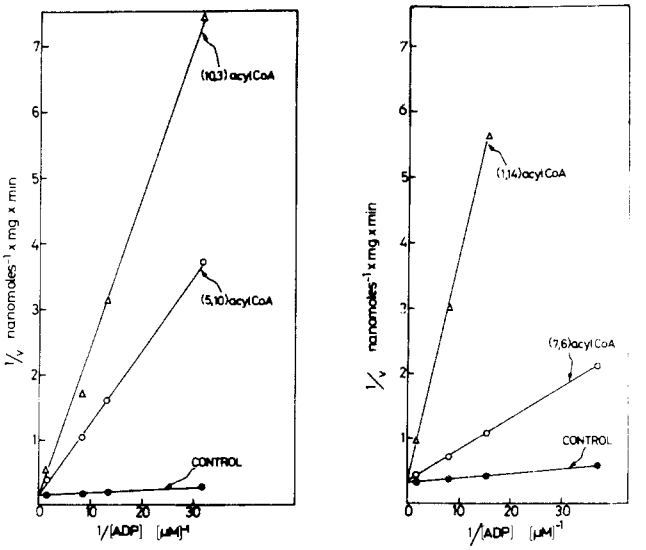


FIGURE 3: Competitive inhibition of ADP translocation by spin-labeled acyl-CoA's. Rat heart mitochondria (3 mg of protein) were preincubated in 5 ml of 110 mM KCl, 0.1 mM EDTA, and 10 mM Tris-sulfate (pH. 7.2) for 1 min at 0° with different concentrations of spin-labeled acyl-CoA's (ranging from 0.3 to 5.4 μM). Then [^{14}C]ADP in 100 μl was added to a final concentration of 8 μM (K_M^{ADP} = 3.2 μM). The incubation lasted for 30 sec at 0° and was stopped by addition of 5 μM carboxyatractyloside followed by rapid centrifugation. The amount of [^{14}C]ADP incorporated in the matrix space was calculated from the amount of [^{14}C]ADP present in the pellet after correction for [^{14}C]ADP in the sucrose space. On the figure only one set of data was plotted for each type of CoA derivative: (A) those obtained with 5.4 μM of (10,3)acyl-CoA and 2.8 μM of (5,10)acyl-CoA; (B) those obtained with 2.2 μM of (7,6)acyl-CoA and with 4.7 μM of (1,14)acyl-CoA. K_i values are, respectively: 0.15 ± 0.04 μM for the (10,3)acyl-CoA; 0.40 ± 0.05 μM for the (7,6)acyl-CoA; 0.22 ± 0.08 μM for the (5,10)acyl-CoA; and 0.15 ± 0.04 μM for the (1,14)acyl-CoA.

will only try to deduce *qualitatively* the origins of these changes. When possible, we measured the anisotropic hyperfine splitting constants $T'_{\parallel}$ and $T'_{\perp}$ by a graphic technique (Seelig, 1970). From these values we calculated the order parameter S, which is a measure of the fluidity. No more detailed esr analysis will be given; it is difficult indeed to attribute a relevant physical parameter to spectra in the case of unresolvable nonhomogeneous spectra.

Membrane Preparations and Assays. Rat liver mitochondria were prepared from 0.27 M sucrose homogenates by the Hogeboom procedure (1955) and washed three times with 0.27 M sucrose buffered with 1 mM Tris-HCl (pH 7.6–7.4). Inner membrane plus matrix and outer membrane particles of rat liver mitochondria were prepared by treatment with 20 mM phosphate (Parsons and Williams, 1967) and inner membrane vesicles devoid of matrix were obtained by further extraction with 20 mM phosphate and passage through a sucrose gradient (Colbeau *et al.*, 1971). Pigeon heart and rat heart mitochondria were prepared in 0.225 M mannitol–0.075 M sucrose, 0.02 M Tris-HCl (pH 7.6), and 0.2 mM EDTA after partial digestion by nagarse (Tyler and Gonze, 1967) and tested in the same buffer with EDTA omitted (MST medium). Protein concentration was measured by the biuret method.

Membrane lipids were extracted according to Dawson *et al.* (1960) (see also Colbeau *et al.*, 1971). The lipids were dispersed in buffer by treatment for 15 min at 0° under argon with a Branson sonifier at maximal output.

The rate of [^{14}C]ADP transport was determined by the direct exchange procedure, the transport being stopped by addition of carboxyatractyloside (gummiferin) as described by Vignais *et al.* (1973a).

The transhydrogenase activity was measured according to Ernster and Lee (1967) with submitochondrial particles (MgATP particles) prepared by sonication of heart mitochondria in the presence of 15 mM $MgCl_2$ and 1 mM ATP (pH 7.5).

Results

Spin-Labeled Long-Chain Acyl-CoA's Are Reversible Inhibitors of the ADP Transport in Mitochondria. As shown in Figure 2, (10,3)acyl-CoA at a concentration of 19 μM totally inhibited the mitochondrial respiration stimulated by a low concentration of ADP (10^{-4} M). This inhibition was reversed when the concentration of ADP was raised to 10^{-3} M (Figure 2A). The inhibition of the ADP-stimulated respiration by (10,3)acyl-CoA was also relieved by uncouplers (Figure 2B), indicating that the acyl-CoA does not interact with the coupling mechanism. In contrast to the CoA derivative, free (10,3)fatty acid, at a concentration of 250 μM, hardly inhibited the ADP-stimulated respiration; such a high concentration of fatty acid had a slight uncoupling effect.

Direct translocation experiments were carried out to ascertain the competitive nature of the inhibition of ADP transport by spin-labeled acyl-CoA's and to measure accurately the inhibition constants. The strictly competitive nature of inhibition is illustrated in Figure 3. Mean average K_i values were 0.15 ± 0.04 μM for the (10,3)acyl-CoA and the (1,14)acyl-CoA, 0.22 ± 0.08 μM for the (5,10)acyl-CoA, and 0.40 ± 0.05 μM for the (7,6)acyl-CoA. These K_i values are similar to those found for natural long-chain acyl-CoA's (Morel *et al.*, 1974), a result which fulfills one of the requirements for the use of spin-labeled molecules as probes

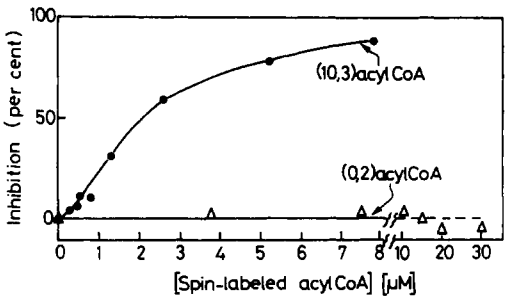


FIGURE 4: Effect of increasing concentrations of (10,3)acyl-CoA and (0,2)acyl-CoA on the rate of ADP translocation in rat heart mitochondria. Same conditions as in Figure 3; rat heart mitochondria, 2.8 mg of protein.

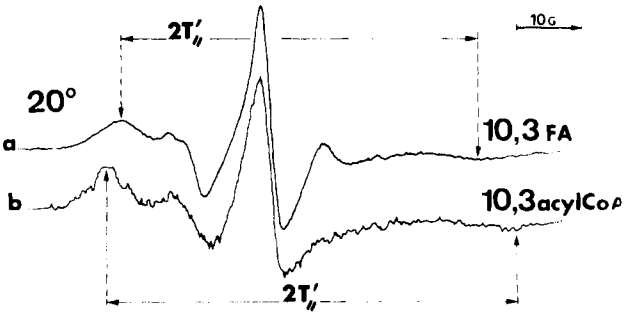


FIGURE 5: Esr spectra of (a) (10,3)FA and (b) (10,3)acyl-CoA in pigeon heart mitochondria. The concentration of mitochondria is 80 mg/ml and corresponds approximately to 8×10^{-5} M of ADP carrier. The concentration of (10,3)acyl-CoA is 5×10^{-5} M. (12,3)Acyl-CoA gives a spectrum identical with that seen with the (10,3)acyl-CoA.

(*cf.* introductory statement). This very high affinity of long-chain acyl-CoA is in sharp contrast with the poor inhibitory effect of short-chain acyl-CoA. For instance, the (0,2)acyl-CoA (C_5) had no inhibitory effect on ADP transport up to a concentration of 30 μM (Figure 4).

Long-chain acyl-CoA's inhibit a number of enzymes (*cf.* Rydström, 1972). In the inner mitochondrial membrane the two functions which are the most sensitive to palmityl-CoA are the ADP carrier (Pande and Blanchaer, 1971; Morel *et al.*, 1974) and the pyridine–nucleotide transhydrogenase (Rydström, 1972), the K_i being lower than 1 μM in both systems. This interaction of a spin-labeled acyl-CoA with mitochondrial membranes could be attributed to either the ADP carrier or to the transhydrogenase. To discriminate between these two possibilities, it was necessary to examine the effect of specific ligands on the displacement of the spin-labeled acyl-CoA. ADP and inhibitors of the ADP transport, atractyloside and carboxyatractyloside, interact specifically with the ADP carrier (*cf.* Vignais *et al.*, 1973b, for a review); NAD^+ and $NADP^+$ are substrates of transhydrogenase. It was necessary to check, first, the possible occurrence of cross-reactivity. No inhibition of the transhydrogenase activity in MgATP particles by carboxyatractyloside at concentrations as high as 60 μM was found. On the other hand, the transport of ADP was competitively inhibited by NADP and NAD^+, the inhibitory effect of NAD^+ being 3 to 4 times less than that of $NADP^+$; the K_i for $NADP^+$ was of the order of 100 μM. This inhibition of the ADP transport by $NADP^+$ will be taken into account when we discuss the displacement of bound spin-labeled acyl-CoA by $NADP^+$ as related to the transhydrogenase or to the ADP carrier.

Basic Spin Label Experiments and Choice of Mitochondrial Preparations. Figure 5 shows the differences between the esr spectra of (10,3)FA and (10,3)acyl-CoA in pigeon heart mitochondria. This is in agreement with data presented above showing that spin-labeled acyl-CoA interacts with membrane proteins. These interactions can be detected with a sufficient accuracy provided that the ratio of acyl-CoA to protein is maintained at a low level. The corresponding $T'_{\parallel}$ values are given in Table I. The differences between the $T'_{\parallel}$ values obtained with (10,3)FA and (10,3)acyl-CoA increase with temperature. The consequences of this temperature dependence will be discussed later.

In order to find the best system to detect by esr the binding of acyl-CoA's to membrane proteins we tested various mitochondrial preparations. Three types of preparations were found suitable: (1) pigeon heart mitochondria; (2) rat heart mitochondria; and (3) vesicles of inner membrane + matrix from rat liver mitochondria.

Table I: Spectral Characteristics of (10,3)FA and (10,3)-Acyl-CoA in Rat Heart Mitochondria at Various Temperatures.[a]

	Temp (°C)					
	−18	−10	0	10	20	30
$T'_{\parallel}$ (FA) (G)	33.0	31.7	30.5	28.4	26.8	25.5
$T'_{\parallel}$ (acyl-CoA) (G)	33.0	32.5	32.0	31.5	31.0	(29.3)
$\Delta T'_{\parallel}$ (G)	0	0.8	1.5	3.1	4.2	(3.8)

[a] The sample composition is given in the legend of Figure 6.

Whole mitochondria from rat liver showed no clear difference between the spectra of (10,3)FA and of (10,3)acyl-CoA intercalated in these mitochondria. No difference was observed with outer membrane vesicles from rat liver mitochondria. On the other hand, characteristic differences were seen with vesicles of inner membrane + matrix. Since in liver mitochondria the area corresponding to the outer surface of mitochondria is not very much smaller than the area of the inner membrane and, furthermore, since the outer membrane has a high phospholipid content (Colbeau *et al.*, 1971) a large fraction of the (*m,n*)acyl-CoA molecules may reside in the outer membrane. This may explain why heart mitochondria, having a small outer membrane surface compared to the inner membrane surface, constitute a better material than liver mitochondria.

Rat heart mitochondria had to be preincubated with ferricyanide to avoid spin reduction. Pigeon heart mitochondria did not require pretreatment with ferricyanide; this is possibly due to the low content of pigeon heart mitochondria in endogenous substrates. Inner membrane + matrix vesicles from rat liver mitochondria could also be used without ferricyanide. This feature makes them a good candidate for this type of experiment. However, it should be kept in mind that the preparation of the inner mitochondrial membrane may bring serious perturbation in the network arrangement of the inner membrane.

If these differences are due to binding to membrane protein, one predicts (1) that in pure lipid bilayer there will be no difference between the spectra obtained with spin-labeled fatty acid and with its corresponding CoA derivative

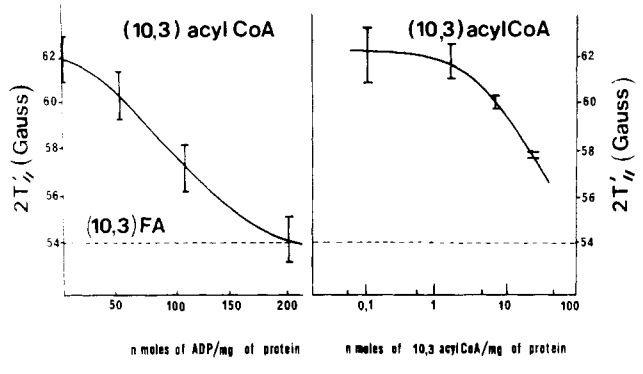


FIGURE 6: Dependence of $T'_{\parallel}$ measured with (10,3)acyl-CoA in heart mitochondria on: (a) concentration of ADP, (b) concentration of (10,3)acyl-CoA. Experiments were done at room temperature. The dotted line shows $2T'_{\parallel}$ for (10,3)FA (which is independent of ADP concentration) for comparison.

and (2) that specific ligands of the ADP carrier will perturb spectrum b. These different points will be developed in the following sections.

Specific Displacements of Bound (10,3)Acyl-CoA by Ligands of the ADP Carrier. The fact that long-chain acyl-CoA's competitively inhibit the transport of ADP across mitochondrial membranes implies that bound acyl-CoA is displaced by excess ADP. In fact the spectrum of (10,3)acyl-CoA bound to rat heart mitochondria is markedly modified upon addition of ADP; in contrast, that of (10,3)FA was not altered by ADP addition. At high concentrations of ADP, the modified (10,3)acyl-CoA spectrum tends toward a limit shape which is not significantly different from that of the bound (10,3)FA; under these conditions the $T'_{\parallel}$ value measured with (10,3)acyl-CoA in heart mitochondria was close to that obtained with (10,3)FA (Figure 6a).

Table II summarizes the effects of a number of nucleotides and specific inhibitors of the ADP transport on the displacement of the low-field peak of bound (10,3)acyl-CoA. A marked shift was obtained upon addition of ADP and ATP; in contrast AMP, GDP, and UDP were without effect. This observation is in line with the fact that ADP and ATP, but not UDP, GDP, or AMP, are substrates for the ADP carrier (Pfaff and Klingenberg, 1968; Winkler *et al.*, 1968; Duée and Vignais, 1969). In the experiment of Figure 6, the (10,3)acyl-CoA was used at a concentration of 10^{-4} M, and 70- to 80-fold excess ADP was required to bring about a half-maximum change of the low-field peak. The ratio of the concentration of ADP to that of (10,3)acyl-CoA was about 75:1. This is comparable to the ratio (≈40:1) of the K_M^{ADP} (3-7 μM) for ADP transport to the K_i (0.10-0.15 μM) related to the inhibition of ADP transport by (10,3)acyl-CoA. This effect of ADP is reversible, *i.e.* when ADP is washed out, again an immobilized spectrum is observed.

Specific inhibitory ligands such as atractyloside and carboxyatractyloside displaced bound (10,3)acyl-CoA. However, bongkrekic acid, at concentrations largely exceeding those which are fully inhibitory, was without effect unless ADP was present; at the concentration used (1 mM), ADP by itself was unable to induce a significant shift of the low-field peak. This recalls the enhancing effect of ADP on the displacement by bongkrekic acid of bound atractyloside or carboxyatractyloside (Vignais *et al.*, 1973a).

Table II: Effect of Various Nucleotides and Inhibitors of ADP-Translocase on the Esr Spectrum of (10,3)Acyl-CoA in Heart Mitochondria.[a]

	Concn (nmol/mg of Mitochondria Protein)	Effect[b]
ADP	100	++
ATP	100	++
Carboxyatractyloside	2	++
Atractyloside	4	++
Bongkrekic acid	10	−
Bongkrekic acid + ADP	10 + 20	++
AMP	100	−
GDP	100	−
UDP	100	−
NAD	100	+
NADP	100	++

[a] Approximate receptor site concentration: 0.5×10^{-4} M (~1 nmol/mg of protein); concentration of (10,3)acyl-CoA, 10^{-4} M. Membranes were previously resuspended at pH 6.6 before use of bongkrekic acid. [b] ++ means that the typical spectrum of (10,3)acyl-CoA in mitochondria approaches the spectrum of (10,3)FA in mitochondria; + means only a small change; − means no change.

$NADP^+$ used at the same concentration as ADP had an effect on the line shapes of the esr spectra similar to that of ADP. NAD^+ was less effective than $NADP^+$. The effect of these latter two may be in part related to the competitive inhibition of $NADP^+$ and NAD^+ for ADP binding to the ADP carrier (see above); the smaller affinity (higher K_i) shown by NAD^+ would explain a lower efficiency of NAD^+ for modifying the spectral line shapes.

When rat heart mitochondria were preincubated at 65° for 5 min, (10,3)acyl-CoA gave the same immobilized spectrum as in untreated mitochondria. However, ADP was unable to shift the low-field peak as was the case for intact mitochondria. Likewise submitochondrial particles obtained by sonication from heart mitochondria could bind (10,3)acyl-CoA, but the bound (10,3)acyl-CoA was not displaced by ADP. These latter results may be interpreted by a direct interaction of (10,3)acyl-CoA with a heat-labile protein whose conformation is modified by sonication. A modification of the titration curves for atractyloside or carboxyatractyloside after sonication of mitochondria has also been reported (Vignais *et al.*, 1973a).

All these data taken together indicate that once (10,3)acyl-CoA is incorporated in heart mitochondrial inner membrane it interacts preferentially with a protein belonging to the ADP carrier and bearing specific receptor sites for ADP, ATP, and specific inhibitors.

Effect of the (10,3)Acyl-CoA Concentration in Heart Mitochondria on the Line Shape of the Esr Spectra. K_i measurements (see above) do not provide a direct determination of the affinity constants of the spin-labeled acyl-CoA to the ADP carrier. In order to find conditions where most of the spin label is bound to this membrane protein, the concentration of spin label was varied over a wide range. At very high levels of (10,3)acyl-CoA (or (12,3)acyl-CoA), three sharp lines appeared, corresponding to the spin label in water.

Figure 6b shows $T'_{\parallel}$ values *vs.* (10,3)acyl-CoA concentration. The shape of the "immobilized" spectrum is inde-

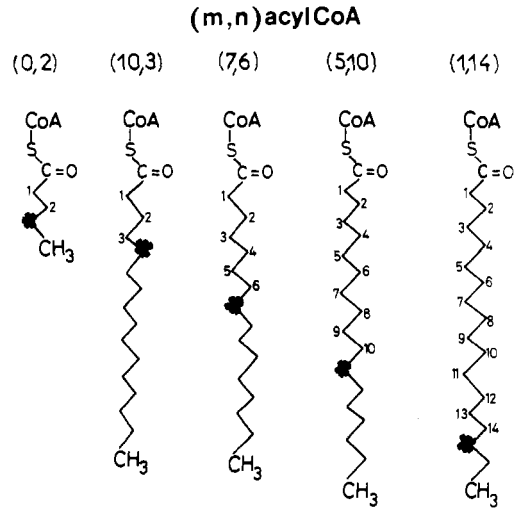


FIGURE 7: Schematic representation of various spin-labeled acyl-CoA's synthesized, showing the position of the probe relative to the polar head group.

pendent of the (10,3)acyl-CoA concentration for ratios of acyl-CoA (nanomoles) to protein (milligrams) below one. It may be inferred from data of Figure 6 that (10,3)acyl-CoA completely binds to mitochondria when added to mitochondria at a ratio (nanomoles/milligrams of protein) lower than one. Since heart mitochondria contain about 1 nmol of ADP carrier per mg of protein—as deduced from the amount of bound carboxyatractyloside (Vignais *et al.*, 1972)—it follows that the molar ratio of acyl-CoA to ADP carrier is lower than one.

In Figure 5, spectrum b corresponds to (10,3)acyl-CoA bound to mitochondria and not to a mixture of (10,3)acyl-CoA bound and unbound. This remark enabled us to discuss below the fluidity of the direct lipid environment of the ADP carrier.

The same study of the line shapes *vs.* spin-label concentration was done with other (*m,n*)acyl-CoA molecules added to heart mitochondria. We shall see now that only (10,3)-, (12,3)-, and (7,6)acyl-CoA allow an estimation by esr of the acyl-CoA affinity for the ADP carrier.

When the Nitroxide Is Moved down the Hydrocarbon Chain of the Spin-Labeled Acyl-CoA the Typical Protein Interactions Disappear. Results obtained with (10,3)acyl-CoA or (12,3)acyl-CoA have indicated that these molecules interact with the ADP carrier. Other spin-labeled acyl-CoA's, differing in the position of the nitroxide radical on the acyl chain, were tested: (7,6)acyl-CoA, (5,10)acyl-CoA, and (1,14)acyl-CoA (Figure 7). All of them inhibit the ADP transport with a K_i value in the range of 0.1–0.4 μM (Figure 3). Esr spectra of the different acyl-CoA's incorporated at low levels in rat heart mitochondria have been taken at several temperatures.

At 20° (and also at 30°) differences between the esr spectra of (*m,n*)FA and (*m,n*)acyl-CoA can be seen with the molecules labeled on the 10,3 and the 7,6 positions (Figures 5 and 8). At 0° differences between the esr spectra of the (*m,n*)FA and (*m,n*)acyl-CoA can be seen with the molecules labeled on the (10,3), (7,6), and also to some extent on the (5,10) positions. No difference is seen when the label is on the (1,14) position.

For all temperatures and labels studied, the differences between the spectra of (*m,n*)acyl-CoA and (*m,n*)FA were abolished by addition of either ADP or carboxyatractyloside. An immobilized fraction of (1,14)FA might be obscured by a comparable fraction of (1,14)FA freely rotating. To make sure that our sample with the (1,14)acyl-CoA did not contain any immobilized fraction we therefore examined the effect of ADP on (1,14)acyl-CoA using a very low level of (1,14)acyl-CoA ($\simeq$0.1 nmol/mg of protein). Esr spectra with or without ADP were identical. It follows that the binding of (1,14)acyl-CoA to ADP carrier cannot be detected by esr.

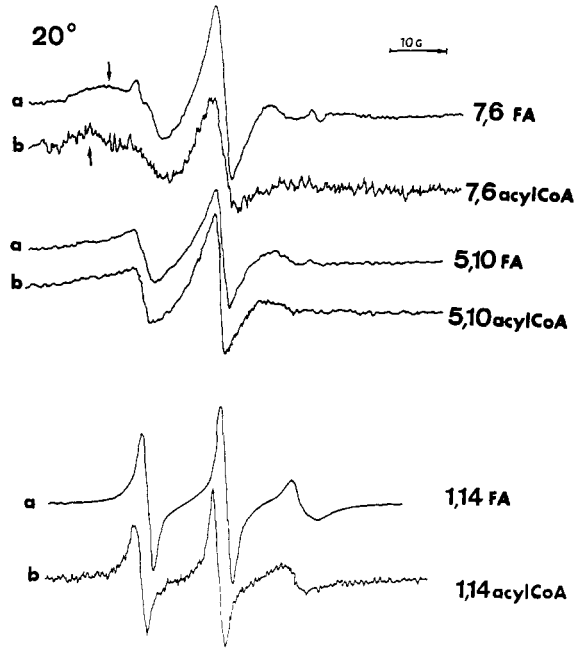
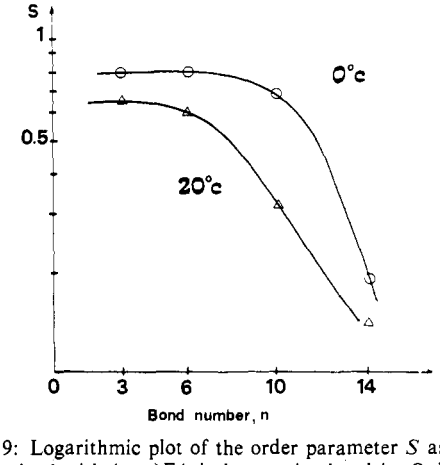


FIGURE 8: Comparison of the esr spectra of fatty acids and acyl-CoA spin labels in heart mitochondria; concentration of mitochondria: 80 mg/ml or approximately 8 × 10^{-5} M of ADP carrier. Concentrations of (7,6)acyl-CoA, (5,10)acyl-CoA, and (1,14)acyl-CoA were 7 × 10^{-6}, 5 × 10^{-5}, and 2 × 10^{-5} M, respectively.



FIGURES 9: Logarithmic plot of the order parameter S as a function of n determined with (*m,n*)FA in heart mitochondria. Only (5,10)FA exhibits a large change of the order parameter between 0 and 20°.

Additional Information on the Mitochondrial Inner Membrane Fluidity. To be discussed safely the previous results require some additional information concerning membrane fluidity. From the spectra of the spin-labeled fatty acids incorporated in rat heart mitochondria, values of the order parameter S have been estimated and plotted *vs.* the position of the nitroxide radical along the hydrocarbon chain (Figure 9).

The discontinuous shape of the curve, characterized by a plateau followed by an abrupt decrease of S, has been observed in other biological membranes (Seelig and Hasselbach, 1971; Eletr *et al.*, 1973; Sackmann *et al.*, 1973). The important point to note is that the (5,10)FA experiences *at 0°* relatively slow *rotational motion* characterized by a high order parameter. But *at 20° the same probe is in a region of high fluidity*. In the same range of temperature, the (10,3)FA and (7,6)FA always have high-order parameters, whereas the (1,14)FA always has a lower order parameter.

The variations of the order parameter determined with the (5,10)FA reveal a broad temperature transition occurring around 10°. Although order parameters for correlation times cannot be measured easily with spin-labeled acyl-CoA's, an abrupt change around 10° in the overall shape of the spectra of (5,10)acyl-CoA in mitochondria strongly suggests that this latter molecule also detects the phase transition in the membrane.

Spin-Labeled Acyl-CoA's Are Readily Incorporated into Sonicated Dispersions of Lipid Extracts. The (0,2)FA and the (0,2)acyl-CoA only give rise to esr spectra typical of these spin labels tumbling rapidly in water. All the other spin-labeled molecules are readily incorporated into lipid dispersions. Spectra of the (10,3)FA and the (10,3)acyl-CoA differ slightly, the fatty acid being slightly more mobile than the acyl-CoA. This difference can account for the small difference between the spectra of (10,3)FA and (10,3)acyl-CoA when the latter is chased from ADP carrier by other ligands.

The overall shape of these various spectra is close to the shape of the spectra obtained with the spin-labeled fatty acids (*m,n*)FA once incorporated into heart mitochondria. One would not expect complete identity, since sonicated dispersions of lipids represent only a crude approximation of the lipid structure occurring in the native membrane.

To estimate the level of incorporation of long-chain spin-labeled acyl-CoA's per phospholipid molecule, we took advantage of the fact that the sharp high-field peak of the free acyl-CoA does not overlap the corresponding peak of the acyl-CoA incorporated into the membrane (except to some extent in the case of the (1,14)acyl-CoA). Therefore, for a given concentration of spin label, the incorporation can be measured from the magnitude of the high-field peak of the "free" acyl-CoA. When a given amount of (*m,n*)acyl-CoA (5×10^{-5} M) was mixed with sonicated dispersions of mitochondrial lipids (5×10^{-4} M in phospholipids) the ratio of membrane-bound acyl-CoA to the aqueous acyl-CoA was 15 for (10,3)acyl-CoA, 5 for (7,6)acyl-CoA, 38 for (5,10)acyl-CoA, and 40 for (1,14)acyl-CoA. Solubility in the lipids is related to the chain length but is also influenced by the position of the oxazolidine group which has a double bond like effect. Solubility in the hydrophobic part of the membrane parallels the inhibitory effect of acyl-CoA's on the ADP carrier (*cf.* Morel *et al.*, 1974). It is striking to compare the small differences in solubility observed with the various spin-labeled acyl-CoA's and the small differences between their inhibitor constants of the ADP transport; for example, (7,6)acyl-CoA is the least soluble and at the same time the least efficient inhibitor of the tested spin-labeled long chain acyl-CoA's.

High levels of incorporation can be obtained easily. When as much as 0.1 mol of spin-labeled acyl-CoA per mol of phospholipid was incorporated the shape of the spectra due to incorporated spin labels was modified by spin–spin interactions. This is especially visible for the (1,14)acyl-CoA spectra. These spectra give a possible way to estimate the lateral diffusion constant of acyl-CoA in mitochondrial membranes. Unfortunately, high concentrations of acyl-CoA have a detergent effect.

Discussion

(1) Specificity of the Probing of the ADP Carrier by Spin-Labeled Acyl-CoA. Long-chain spin-labeled acyl-CoA molecules inhibit the ADP carrier system. The inhibitor constants of spin-labeled acyl-CoA's determined by measuring the rate of [^{14}C]ADP entry into mitochondria are similar to those obtained with ordinary long-chain acyl-CoA's (Morel *et al.*, 1974). Thus, the bulky oxazolidine ring does not interfere with the interaction of the acyl-CoA with the ADP carrier. This was a perequisite for the use of long-chain acyl-CoA's as probes of the ADP carrier.

The primary aim of this investigation was to explore the microenvironment of the ADP carrier in the inner mitochondrial membrane with spin-labeled acyl-CoA's and to probe the dimension and the mobility of the mitochondrial ADP carrier for which long-chain acyl-CoA's are strong competitive inhibitors. However, another protein, the pyridine nucleotide transhydrogenase, also located in the inner membrane, is inhibited by palmityl-CoA (Rydström *et al.*, 1973). Long-chain spin-labeled acyl-CoA's are likewise inhibitory of the ADP transport and of the transhydrogenase. This, therefore, raised the problem of the discrimination of interactions of spin-labeled acyl-CoA with the ADP carrier and the transhydrogenase.

Ligands which interact with the ADP carrier or the transhydrogenase were used to study this problem. NAD^+ and $NADP^+$ displaced bound acyl-CoA, but they are not specific for transhydrogenase because they inhibit the ADP transport and therefore interact with the ADP carrier. On the other hand, carboxyatractyloside, a potent inhibitor of the ADP carrier, has no effect on the activity of the transhydrogenase at concentrations 60 times higher than those required to inhibit totally this ADP transport. Carboxyatractyloside completely displaced bound acyl-CoA. Juntti *et al.* (1970) have shown from experiments based on trypsin inactivation that transhydrogenase is located near the inner side of the inner mitochondrial membrane. This is in line with the fact that transhydrogenase in the inner membrane of intact mitochondria is not readily accessible to added NAD^+ and $NADP^+$, but becomes accessible after sonication which produces inverted membrane vesicles. Furthermore, it is known that acyl-CoA's by themselves do not cross the inner mitochondrial membrane. So it is likely that the mitochondrial transhydrogenase is not readily accessible to long-chain spin-labeled fatty acids.

(2) Orientation of the Spin-Labeled Acyl-CoA on the ADP Carrier. The ability of acyl-CoA to penetrate and to stay in the membrane is a function of the length of the hydrocarbon chain. Short-chain acyl-CoA's have a lower affinity for the ADP carrier (Morel *et al.*, 1974) and the spin-labeled (0,2)acyl-CoA failed to interact with the ADP carrier as well as to penetrate artificial phospholipid vesicles. Consequently it seems that the hydrocarbon chain is required to anchor the acyl-CoA molecule in the lipid phase of the membrane. On the other hand, the considerable enhancement by CoA of the inhibitory effect of long-chain fatty acids on the ADP transport (Vaartjes *et al.*, 1972) and the presence of an adenylic residue in the acyl-CoA molecule strongly suggest an interaction between the ADP binding site in the ADP carrier and the CoA moiety of the

acyl-CoA. One may imagine that the carrier possesses a hydrophobic region interacting with the acyl chain of the acyl-CoA and a hydrophilic region which binds the CoA moiety of the acyl-CoA. Because of polarity analogy, the CoA may mimic the ribose diphosphate portion of ADP and the acyl chain, the adenine ring of ADP. Spin-labeled fatty acids, at very high concentrations (one to two orders of magnitude in excess with respect to those of the corresponding acyl-CoA's), could interact with the ADP carrier and inhibit its activity (see Vaartjes *et al.*, 1972); however, under these conditions, free fatty acids most likely interact with other hydrophobic proteins of the inner mitochondrial membrane in an unspecific manner. It may be recalled here that specific interaction of spin-labeled acyl-CoA's with the ADP carrier requires the use of concentrations of acyl-CoA just sufficient to saturate the carrier units and that the specificity of this interaction has been checked by displacement of bound spin-labeled acyl-CoA's by specific ligands of the ADP carrier.

We have shown that the (10,3)acyl-CoA and the (7,6)acyl-CoA can be used to detect between 0 and 30° the binding of acyl-CoA molecules to the ADP carrier. The (5,10)acyl-CoA barely detects association at 0° and none above 0° and the (1,14)acyl-CoA is unable to detect association at or above 0°. The differences in the ability to detect the ADP carrier with the various spin labels can be simply interpreted in terms of a rather short protein not extending completely throughout the bilayer and maintained immobilized once bound to acyl-CoA. This hypothesis will be developed further when considering the different models of acyl-CoA–ADP carrier interactions.

(*3*) *Movements of Spin-Labeled Acyl-CoA's in the Lipid Phase of the Inner Mitochondrial Membrane.* When intercalated in mitochondria at room temperature, both the (1,14)acyl-CoA and (5,10)acyl-CoA molecules give esr spectra identical with those obtained in the same conditions with either free (1,14)FA or (5,10)FA, in spite of the fact that the two acyl-CoA molecules bind to and inhibit the ADP carrier. The esr spectra of the four above-mentioned spin-labeled molecules are of the type observed with fluid lipid bilayers.

Although the spectra of the (7,6)acyl-CoA and of (10,3)acyl-CoA indicate some immobilization due likely to a selective binding to the ADP carrier, they correspond to less immobilization than the spectra given by spin-labeled fatty acids bound to bovine serum albumin. This suggests that, in spite of strong interactions with the ADP carrier (their inhibitor constant is in the range of 10^{-7} M), the nitroxide of the two latter spin-labeled molecules is nevertheless close to a fluid hydrophobic environment.

The differences observed above 0° between the spectra of spin-labeled fatty acids and of acyl-CoA's cannot be attributed to differences in polarity. At low temperature (−18°) the (10,3)FA and (10,3)acyl-CoA give very similar spectra indicative of a complete immobilization of the nitroxide (Table I). If these probes were experiencing a different local polarity, they should give rise to different esr spectra in spite of the complete immobilization of the nitroxide. In consequence the differences in line shapes observed at room temperature are attributed to differences in motion only. This restriction in motion is comparable to what Jost *et al.* (1973) found for spin-labeled fatty acids in close contact with cytochrome oxidase in reconstituted systems. The difference in motion is probably not due to a local difference in lipid composition. In fact, a broad phase transition around

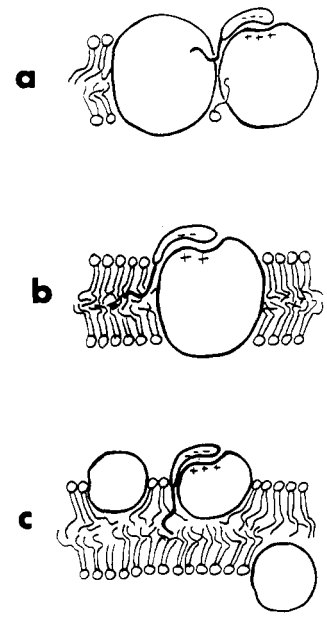


FIGURE 10: Lipid environment of the ADP carrier; different models. See text for discussion.

10° can be detected with both (5,10)FA and (5,10)acyl-CoA using either order parameter plot or simply $T'_{\parallel}$ plots *vs.* temperature so that the lipid composition around the ADP carrier is likely that of the average lipid bilayer of the membrane. In contrast, Stier and Sackmann (1973) found in liver microsomal membranes an extended region of lipids more immobilized around the cytochrome P-450 than in the bulk lipids.

The displacement of bound acyl-CoA in mitochondria by atractyloside, carboxyatractyloside, or ADP is reflected by a change of the esr spectra of the spin-labeled acyl-CoA which now resemble that given by the free fatty acid molecules. This supports the idea that unbound acyl-CoA molecules are free to diffuse away from the protein, and conversely that the ADP carrier is accessible to acyl-CoA from the lipid core of the membrane. Since bound and unbound acyl-CoA's are in equilibrium in the core of the inner mitochondrial membrane, it is inferred that the ADP carrier has at least a common boundary with a fluid lipid bilayer. It has been shown recently that the lipids in the inner mitochondrial membranes of rat liver diffuse rapidly and that only a small fraction of lipids are in a rigid state (P. Devaux *et al.*, manuscript in preparation). On the other hand there exists some evidence of specific protein–protein associations. For instance, enzymes of the respiratory chain are assembled in a definite order (*cf.* Racker *et al.*, 1970); the ADP translocase itself may be close to ATP-synthetase (Bertagnolli and Hanson, 1973) and possibly to other transport proteins (Morel *et al.*, 1974). Whether these are purely statistical contiguities or rigid organized structures cannot be stated at the present time.

(*4*) *Models for Interaction of Spin-Labeled Acyl-CoA with the ADP Carrier.* Our results enable us to distinguish three possible models for interaction of the spin-labeled acyl-CoA with the ADP carrier (Figure 10).

The first model (a) implies a pool of proteins with practically no fluid lipids in close contact. This predicts a strong immobilization all along the hydrocarbon chain of the acyl-CoA molecule. Such an immobilization independent of the position of the probe is in fact seen only with delipidated

mitochondria. Consequently model a can be rejected. Model b implies a protein that spans the lipid bilayer in the vicinity of fluid lipids. One might then expect either an unmodified immobilization or a gradual decrease in immobilization when the probe is moved down the chain which does not fit with experimental results. So we believe that model b is not likely to be correct either. Model c implies binding to a protein that does not cross completely the lipid bilayer. This would explain the abrupt decrease in order parameter when the probe is moved between the seventh and the tenth carbon atoms.

The fact that order parameters measured with spin-labeled fatty acids also decrease rapidly after the tenth carbon bond (Figure 9) suggests that in a similar way many other proteins do not extend completely through the lipid core of the membrane. Cholesterol influences the spectra of spin-labeled fatty acids in model membranes in a similar fashion (Hubbell and McConnell, 1971; Schreier-Mucillo *et al.*, 1973).

This discussion assumes that ADP and acyl-CoA both bind to the ADP carrier. In fact, we do not really know whether the sites of fixation of acyl-CoA and ADP are the same (in spite of the resemblance of CoA with ADP). One can imagine as an alternative that the ADP transport involves two molecular entities (an ADP carrier and a regulatory subunit) and that acyl-CoA binds only to the regulatory subunit.

Acknowledgments

The expert technical assistance of Mrs. J. Chabert is gratefully acknowledged. The authors are also indebted to Dr. C. J. Scandella for critically reading the manuscript.

References

Bertagnolli, B. L., and Hanson, J. B. (1973), *Plant Physiol. 52,* 431–435.

Colbeau, A., Nachbaur, J., and Vignais, P. M. (1971), *Biochim. Biophys. Acta 249,* 462–492.

Dawson, R. M. C., Hemington, N., and Lindsay, D. B. (1960), *Biochem. J. 77,* 226–230.

Duée, E. D., and Vignais, P. V. (1969), *J. Biol. Chem. 244,* 3920–3931.

Eletr, S., Zakim, D., and Vessey, D. A. (1973), *J. Mol. Biol. 78,* 351–362.

Ernster, L., and Lee, C. P. (1967), *Methods Enzymol. 10,* 738–744.

Hogeboom, G. (1955), *Methods Enzymol. 1,* 16–19.

Hubbell, W. L., and McConnell, H. M. (1971), *J. Amer. Chem. Soc. 93,* 314–326.

Jost, P. C., Capadil, R. A., Van der Koi, G., and Griffith O. H. (1973), *J. Supramol. Struct. 1,* 269–280.

Jost, P., Waggoner, A. S., and Griffith, O. H. (1971), *in* Structure and Function of Biological Membranes, Rothfield, L., Ed., New York, N.Y., Academic Press, pp 84–144.

Juntti, K., Torndal, U. B., and Ernster, L. (1970), *in* Electron Transport and Energy Conservation, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Edd., Bari, Italy, Adriatica Editrice, pp 257–271.

Keana, J. W., Keana, S. B., and Beetham, D. (1967), *J. Amer. Chem. Soc. 89,* 3055–3056.

Keith, A. D. Sharnoff, M., and Cohn, G. E. (1973), *Biochim. Biophys. Acta 300,* 379–419.

McConnell, H. M., and McFarland, B. G. (1970), *Quart. Rev. Biophys. 3,* 91–136.

Morel, F., Lauquin, G., Lunardi, J., Duszynski, J., and Vignais, P. V. (1974), *FEBS Lett. 39,* 133–138.

Pande, S. V., and Blanchaer, M. C. (1971), *J. Biol. Chem. 246,* 402–411.

Parsons, D. F., and Williams, G. R. (1967), *Methods Enzymol. 10,* 443–448.

Pfaff, E., and Klingenberg, M. (1968), *Eur. J. Biochem. 6,* 66–79.

Pullman, M. E. (1973), *Anal. Biochem. 54,* 188–198.

Racker, E., Burstein, C., Loyter, A., and Christiansen, R. O. (1970), *in* Electron Transport and Energy Conservation, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Bari, Italy, Adriatica Editrice, pp 235–252.

Rydström, J. (1972), *Eur. J. Biochem. 31,* 496–504.

Rydström, J., Hoek, J. B., Alm, R., and Ernster, L. (1973), *in* Mechanisms in Bioenergetics, Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E., Silliprandi, N., Ed., New York, N.Y., Academic Press, pp 579–589.

Sackmann, E., Träuble, H., Galla, H. J., and Overath, P. (1973), *Biochemistry 12,* 5360–5368.

Schreier-Mucillo, S., March, D., Dugas, H., Schneider, H., and Smith, I. C. P. (1973), *Chem. Phys. Lipids 10,* 11–27.

Seelig, J. (1970), *J. Amer. Chem. Soc. 92,* 3881–3887.

Seelig, J., and Hasselbach, W. (1971), *Eur. J. Biochem. 21,* 17–21.

Sellinger, Z., and Lapidot, Y. (1966), *J. Lipid Res. 7,* 174–175.

Stier, A., and Sackmann, E. (1973), *Bichim. Biophys. Acta 311,* 400–408.

Tyler, D. D., and Gonze, J. (1967), *Methods Enzymol. 10,* 75–77.

Vaartjes, W. J., Kemp, A., Jr., Souverijn, J. H. M., and Van den Bergh, S. G. (1972), *FEBS Lett. 23,* 303–308.

Vignais, P. V., Vignais, P. M., and Defaye, G. (1973a), *Biochemistry 12,* 1508–1519.

Vignais, P. V., Vignais, P. M., Defaye, G., Chabert, J., Doussière, J., and Brandolin, G. (1972), *in* Biochemistry and Biophysics of Mitochondrial Membranes, Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E., and Siliprandi, N., Ed., New York, London, Academic Press, pp 447–464.

Vignais, P. V., Vignais, P. M., Lauquin, G., and Morel, F. (1973b), *Biochimie 55,* 763–778.

Vignais, P. V., and Zabin, I. (1958), *Biochim. Biophys. Acta 29,* 263–269.

Weidmann, S. W., Drysdale, G. R., and Mildvan, A. S. (1973), *Biochemistry 12,* 1874–1883.

Winkler, H. H., Bygrave, F. L., and Lehninger, A. L. (1968), *J. Biol. Chem. 243,* 20–28.