Spin-Labeled Acyl Atractyloside as a Probe of the Mitochondrial Adenosine Diphosphate Carrier. Asymmetry of the Carrier and Direct Lipid Environment†

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ABSTRACT: A number of spin-labeled acyl derivatives of atractyloside, (*m,n*)acyl-ATR (general formula: CH_3-$(CH_2)_m$CX$(CH_2)_n$COO-ATR, where X is an oxazolidine ring containing a nitroxide), have been synthesized. As shown by electron spin resonance (ESR) spectra of spin-labeled acyl-ATR, the nitroxide placed on the acyl chain interacts with the diterpene residue of the atractyloside moiety when incorporated in liposomes. Spin-labeled acyl-ATRs were used to probe the ADP carrier in heart mitochondria. They inhibit ADP transport with the same efficiency as unlabeled acyl-ATRs. The inhibition is a mixed competitive and noncompetitive inhibition. The inhibitor constant is close to 10^{-7} M. The long chain acyl-ATRs ((10,3)-, (7,6)-, (7,8)-, and (5,10)acyl-ATRs) and also the short chain (0,2)acyl-ATR, when added at low concentrations to heart mitochondria, give rise to more immobilized ESR spectra than when added to liposomes. Immobilization is stronger for the first three molecules of the series. The (1,14)acyl-ATR, which possesses a nitroxide almost at the end of the acyl chain near the terminal methyl, gives rise to a spectrum corresponding to a high degree of fluidity. Upon addition of atractyloside or of other specific ligands, spin-labeled long-chain acyl-ATRs bound to the ADP carrier are displaced from their binding site toward the lipid phase of the mitochondrial membrane and the short chain (0,2)acyl-ATR is released into the aqueous phase. Spin-labeled long-chain acyl-ATRs do not show any evidence of binding to a protein when incubated with "inside out" submitochondrial particles, in spite of the fact that these particles are able to transport ADP. These results are discussed with respect to the size and the asymmetry of the ADP carrier in the mitochondrial membrane and the mechanism of ADP transport.

In a previous paper (Devaux et al., 1975), spin-labeled acyl-CoAs[1] were used to study the direct lipid environment of the mitochondrial ADP carrier. It was shown that spin-labeled long-chain acyl-CoAs having a nitroxide radical on the acyl moiety have the same inhibitory properties on ADP transport as natural long-chain acyl-CoAs. Such molecules were used to probe the ADP carrier of rat (or pigeon) heart mitochondria. An interesting finding brought about by this study was that, when the probe is moved down the hydrocarbon chain of acyl-CoA, all binding features tend to disappear from the ESR spectra. This was taken to mean that the protein in its inactive state, i.e., bound to acyl-CoA, does not extend through the membrane or is very narrow in the middle of the bilayer. The same data were consistent with the view that, very close to the ADP carrier, fluid lipids can be found.

The present work has been carried out with a different amphipathic spin label, made of a spin-labeled fatty acid linked to atractyloside, a well-known inhibitor of ADP transport in mitochondria (for review, cf. Vignais, 1976). A number of long-chain spin-labeled acyl-ATRs have been synthesized and used to probe the ADP carrier either in whole mitochondria or in "inside out" submitochondrial particles. The results confirm and extend those obtained with spin-labeled long-chain acyl-CoAs. In addition, it will be shown with spin-labeled acyl-ATRs incorporated into liposomes that the spin label on the acyl chain can probe the rigid diterpene residue of the atractyloside moiety of acyl-ATRs. By this means, the penetration of the diterpene residue of acyl-ATR in the lipid bilayer can be evaluated.

Materials and Methods

Membrane Preparations and Assays. Rat heart mitochondria were prepared in 0.225 M mannitol, 0.075 M sucrose, 0.02 M Tris-HCl (pH 7.4), and 0.2 mM EDTA after partial digestion by Nargase (Tyler and Gonze, 1967). Submitochondrial particles were prepared by sonication of beef heart mitochondria in a medium containing sucrose (0.25 M), Tris buffer (3 mM), $MgCl_2$ (15 mM), ADP (5 mM), oligomycin (5 μg per mg of protein) at pH 7.4. The mitochondrial suspension was exposed to sonic oscillations in a Branson sonifier at maximum output for six periods of 30 s at 2–4 °C, separated by intervals of 1 min. Unbroken mitochondria were removed by centrifugation at 15 000 rpm (Spinco rotor 30) for 15 min. The sonic particles were recovered from the supernatant by centrifugation at 30 000 rpm (Spinco rotor 30) for 60 min. The pellet was resuspended in 0.25 M sucrose–3 mM Tris-HCl (pH 7.4). As shown by electron microscopy after negative staining, it consisted of vesicles of 0.15–0.30 μm diameter, most of which (more than 90%) had an inverted orientation of the membrane with stalked particles corresponding to the F_1-ATPase facing the medium.

Lipids from beef heart mitochondria were extracted ac-

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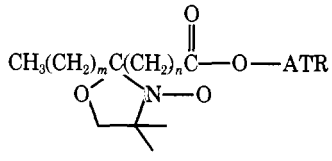
[1] Abbreviations used: acyl-ATR, acyl atractyloside; acyl-CoA, acyl-coenzyme A; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ESR, electron spin resonance; Mops, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

cording to the method of Dawson et al. (1960) as adapted by Colbeau et al. (1971) for mitochondria. Liposomes loaded with spin-labeled acyl-ATR were prepared at room temperature by dispersion of dry lipids (final concentration, 40 mM) and acyl-ATR in 0.12 M KCl–20 mM Tris-HCl (pH 7.4) with small glass beads (molar ratio of spin label to lipid, 1:100).

The rate of ADP transport was assayed by the direct exchange procedure (Duée and Vignais, 1969). The transport was stopped by addition of carboxyatractyloside (Vignais et al., 1973).

Synthesis of Acyl-ATR and Spin-Labeled Acyl-ATR. Natural long-chain fatty acids and spin-labeled long-chain fatty acids were coupled to atractyloside, following the same procedure as that described for the synthesis of succinyl-ATR (Brandolin et al., 1974). Spin-labeled fatty acids and the corresponding anhydrides were synthesized according to Hubbell and McConnell (1971). In routine preparations, 25 mg of anhydride was added to 10 mg of atractyloside in 2 mL of pyridine and allowed to react for 48 h at room temperature. Acyl-ATR was purified by chromatography on a thin layer of silica, using chloroform–methanol–acetic acid–water (65:20:2:1, v/v/v/v) as the solvent system. Beside the unreacted atractyloside, two major bands containing atractyloside could be detected by staining with a vanillin reagent (Vignais et al., 1971). The less polar product corresponding to the fast moving band had a strong inhibitory effect on ADP transport in contrast to the other one. It contained palmitoyl and atractyloside residues in a ratio of one to one, as shown by the synthesis of [^{14}C]palmitoyl-[^{3}H]ATR. Further purification of acyl-ATR was achieved by chromatography on a Sephadex G-25 column, equilibrated with 50 mM triethylamine and 50 mM ammonium bicarbonate, 50:50 v:v, pH 8.2. Acyl-ATR was eluted with the same buffer. The general formula of spin-labeled acyl-ATR is:



ESR Measurements. The ESR spectra were recorded using either a Thomson CSF, TSN 254-2, *X* band spectrometer or a Varian E9-century line. Samples in a 100-μL flat quartz cell were mounted in a variable-temperature accessory. Spin labels were incorporated in the mitochondrial membranes by adding the spin label dissolved in water or in a concentrated organic solution. The concentration of spin-labeled acyl-ATR was between 0.3 and 0.5 nmol/mg of protein, and that of mitochondrial protein between 50 and 60 mg/mL. The volume of the spin-labeled acyl-ATR solution was less than 2% of the volume of the mitochondrial suspension. Unless indicated, the final pH was 7.4. At room temperature the ESR signal of the acyl-ATR incorporated in the membranes decreased slowly due to chemical reduction of the nitroxide. Ferricyanide prevented the reduction but also seemed to interfere with the binding of acyl-ATR to the ADP carrier. Consequently to minimize the chemical reduction of the nitroxide, the displacement studies were performed at 0 °C.

Results

Spin-Labeled Acyl-ATRs in Liposomes. Crude liposomes loaded with spin-labeled acyl-ATRs were prepared as described in Materials and Methods. The shape of the ESR spectra was characteristic of a viscous environment, indicating a complete incorporation of the long-chain spin labels in the lipid phase. The same spin labels in water gave rise to the usual three narrow lines. In contrast to the long chain acyl-ATRs, the short-chain derivative—(0,2)acyl-ATR—showed no affinity for lipid vesicles in our experimental conditions.

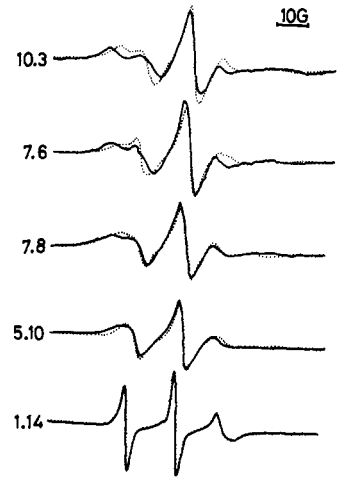


FIGURE 1: Comparison of the ESR spectra of spin-labeled acyl-ATRs (—) and spin-labeled fatty acids (· · ·) in liposomes. Conditions as described in Materials and Methods. The temperature was 25 °C. Note that only the (1,14)acyl-ATR and the (1,14)fatty acid give rise to identical spectra.

The ESR spectra of spin-labeled long-chain acyl-ATRs and those of the corresponding spin-labeled free fatty acids incorporated into liposomes have been compared at temperatures from 0 to 40 °C. Only the (1,14)acyl-ATR and the (1,14)fatty acid gave rise to identical spectra (Figure 1). A small difference could be seen between the (5,10)acyl-ATR and the (5,10)fatty acid. Other atractyloside derivatives—(10,3)-, (7,6)-, and (7,8)acyl-ATRs—exhibited a higher immobilization than the corresponding spin-labeled free fatty acids as judged, for example, from the extreme splittings. Immobilization of the probe in the acyl chain was most likely due to interaction between the acyl chain and the hydrophobic diterpene residue of atractyloside (see Discussion).

Inhibitory and Binding Properties of Acyl-ATRs in Whole Mitochondria and in Sonic Particles ("Inside Out" Particles). Spin-labeled acyl-ATRs were able to inhibit the ADP-stimulated respiration in rat liver mitochondria. The inhibition was partially relieved by increasing concentrations of ADP and totally relieved by FCCP, indicating that the target effect of acyl-ATR was not on the respiratory chain. Actually acyl-ATR, like atractyloside, inhibits ADP transport in mitochondria (Figure 2). However, the inhibition of ADP transport in rat heart mitochondria by short- or long-chain acyl-ATRs was a mixed type inhibition which contrasts with the apparent competitive inhibition caused by atractyloside. The K_i value for acyl-ATR, calculated from the slopes of the lines in Figure 2, was approximately 0.1 μM, which indicates that the inhibitory efficiency of spin-labeled acyl-ATR is similar to that of atractyloside. The inhibitory efficiency and the character of the inhibition caused by acyl-ATR was irrespective of the presence of a nitroxide radical on the fatty acyl moiety.

Binding experiments carried out with rat heart mitochondria showed that (7,6)acyl-[^{3}H]ATR binds to heart mitochondria with no saturation plateau (Figure 3). However, it was possible to distinguish a region of high affinity binding, corresponding to 0.7–0.9 nmol of sites/mg of protein and characterized by a K_d value of about 30 nM. Addition of 200 μM ADP to mitochondria together with (7,6)acyl-[^{3}]ATR did not result in

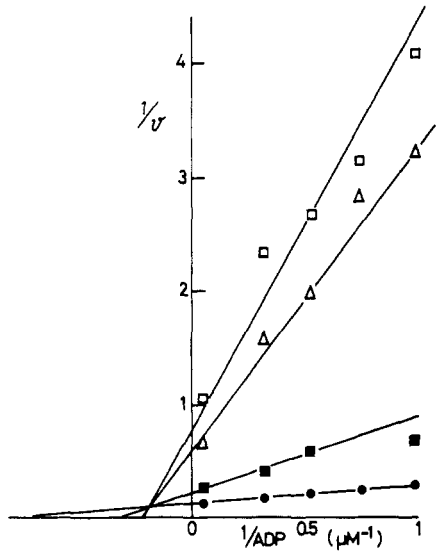


FIGURE 2: Inhibition of ADP transport in rat heart mitochondria by (7,6)acyl-ATR. Mitochondria were preincubated for 5 min at 0 °C in a medium with a final volume of 3 mL containing 0.12 M KCl-20 mM Tris-HCl, pH 7.4, in the absence (●) or presence of (7,6)acyl-ATR. (7,6)Acyl-ATR was used at the following concentrations: 1 μM (□), 0.6 μM (△), 0.3 μM (■). ADP transport was initiated by addition of [^{14}C]ADP and stopped by addition of 8 μM carboxyatractyloside followed by rapid centrifugation. The pellet was dissolved in formamide at 180 °C and the radioactivity was assayed by scintillation counting. Unspecific binding of [^{14}C]ADP was assessed from the radioactivity incorporated in the presence of carboxyatractyloside (8 μM) added before [^{14}C]ADP. v is given in nanomol (mg of protein)$^{-1}$ min^{-1}.

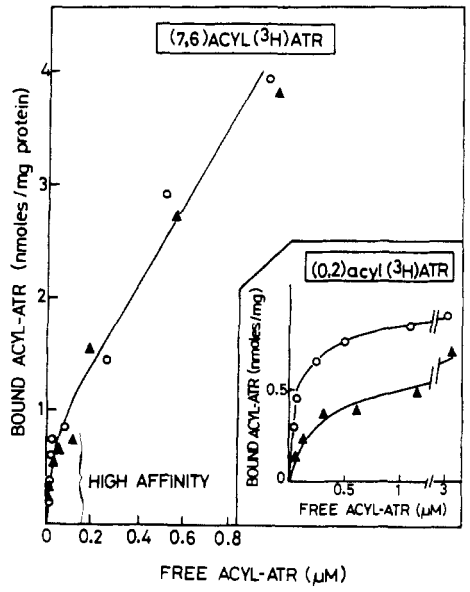


FIGURE 3: Effect of ADP on the binding of (7,6)acyl-[^{3}H]ATR and of (0,2)acyl-[^{3}H]ATR to rat heart mitochondria. Mitochondria (1 mg) were incubated for 45 min at 0 °C in a medium with a final volume of 2 mL containing 0.12 M KCl and 10 mM Mops, pH 7.5, with increasing concentrations of (7,6)acyl-[^{3}H]ATR or (0,2)acyl-[^{3}H]ATR in the absence of ADP (○) or in the presence of 200 μM ADP (▲). After centrifugation, the radioactivity in the pellet was determined by scintillation counting.

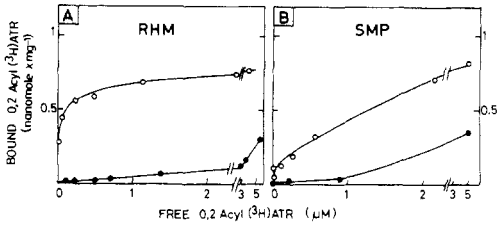


FIGURE 4: Effect of atractyloside on the binding of (0,2)acyl-[^{3}H]ATR to rat heart mitochondria (A) and submitochondrial particles (B). Mitochondria (1 mg of protein) or submitochondrial particles (0.5 mg of protein) were incubated for 45 min at 0 °C with increasing concentrations of (0,2)acyl-[^{3}H]ATR in a medium with a final volume of 2 mL containing 0.12 M KCl and 10 mM Mops, pH 7.5, in the absence (○) or presence (●) of 10 μM atractyloside (ATR). After centrifugation, the radioactivity in the pellet was determined by scintillation counting.

a significant decrease of the bound radioactivity (Figure 3). Furthermore, atractyloside added at a concentration as high as 25 μM, together with (7,6)acyl-[^{3}H]ATR, lowered the bound radioactivity by less than 10%. A similar effect was observed when atractyloside was added to mitochondria preincubated with (7,6)acyl-[^{3}H]ATR. It will be shown in ESR experiments presented later in this paper that, actually, the bound (7,6)acyl-[^{3}H]ATR is displaced by ADP or atractyloside, not to the water phase, but to the lipid core of the mitochondrial membrane.

In contrast with long-chain acyl-ATRs, the binding of the short chain (0,2)acyl-[^{3}H]ATR to rat heart mitochondria was characterized by a well-defined region of high affinity binding (Figure 3, inset). The number of sites was 0.7–0.8 nmol/mg of protein and the K_d value, 25 nM. Furthermore, ADP and atractyloside competed efficiently with (0,2)acyl-[^{3}H]ATR for binding to mitochondria (Figures 3 and 4A).

The beef heart sonic particles used for acyl-ATR binding and inhibition studies were "inside-out" to an extent of 90% (cf. Materials and Methods). When loaded with ADP, they could exchange their internal ADP against added ADP. ADP transport in sonic particles was inhibited by bongkrekic acid, as it is in mitochondria (Henderson et al., 1970; Kemp et al., 1970, 1971); but in agreement with other reports (Shertzer and Racker, 1974; Klingenberg et al., 1974), it was insensitive to atractyloside. It was also insensitive to long- and short-chain acyl-ATRs. In agreement with the inhibition data, the long-chain acyl-[^{3}H]ATR did not bind to sonic particles. But, unexpectedly, the short-chain (0,2)acyl-[^{3}H]ATR was able to bind to sonic particles with a relatively high affinity (K_d = 150 nM) (Figure 4B). However, it must be noticed that the amount of (0,2)acyl-[^{3}H]ATR bound per mg of protein with high affinity was only one-fifth to one-third that found in whole mitochondria. There was a competition between (0,2)acyl-[^{3}H]ATR and atractyloside for binding to sonic particles (Figure 4B) and also between (0,2)acyl-[^{3}H]ATR and bongkrekic acid, which is, like atractyloside, a specific ligand of the ADP carrier. These data indicate that (0,2)acyl-[^{3}H]ATR is able to bind specifically to a small number of sites when added to sonic particles.

ESR Experiments with Spin-Labeled acyl-ATRs. (a) Rat Heart Mitochondria. The ESR spectra of spin-labeled long-chain acyl-ATRs incorporated in mitochondria depended on the position of the nitroxide on the acyl chain. Spectra of the molecules labeled near the polar head group ((10,3)-, (7,6)-, and (7,8)acyl-ATRs) revealed a strong limitation in the motion of the probe (Figure 5). The order parameters were higher than those found when using liposomes (Figure 1). A change of temperature between −10 and +35 °C had little effect on the spectra. When the probe was attached further away from the atractyloside residue, as is the case for the (5,10)acyl-ATR and particularly the (1,14)acyl-ATR, the spectra indicated more fluidity or disorder, although the degree of fluidity or disorder of the corresponding free fatty acid was not reached. Finally

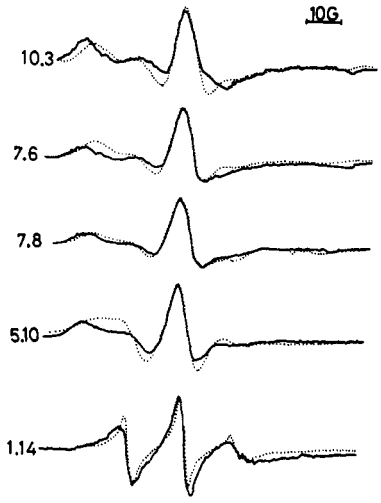


FIGURE 5: ESR spectra of long-chain spin-labeled acyl-ATRs in beef heart mitochondria. Conditions are described in Materials and Methods. The temperature was 25 °C. The dotted traces correspond to the spectra recorded after addition of unlabeled atractyloside to a concentration of 15 nmol/mg of protein. These latter spectra are not totally identical with those obtained with spin-labeled free fatty acids added to beef heart mitochondria.

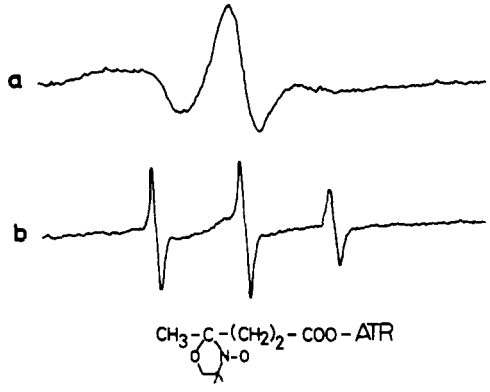


FIGURE 6: Effect of atractyloside on the ESR spectrum of (0,2)acyl-ATR in beef heart mitochondria. Control spectrum is given in a; the spectrum after addition of atractyloside is given in b. Experimental conditions were the same as in Figure 5. Atractyloside was added at a concentration of 15 nmol/mg of protein.

the short-chain (0,2)acyl-ATR strongly interacted with the mitochondrial membrane, although it did not show any affinity for liposomes (Figure 6a). Therefore all acyl-ATR derivatives tested are likely to bind to a mitochondrial protein, presumably the ADP translocator, which carries the binding site of atractyloside. Indeed addition of atractyloside to mitochondria preincubated with spin-labeled long-chain acyl-ATRs rendered the ESR spectra nearly as mobile as those of the corresponding spin-labeled free fatty acids in the mitochondrial membrane (Figure 5, dotted lines); however, they were not totally identical. The most dramatic effect was obtained with the short-chain (0,2)acyl-ATR, which experienced a very rapid tumbling upon addition of atractyloside (Figure 6b). A titration of this effect showed that about 20 and 40% of the bound acyl-ATR were released by addition of 1.5 and 5 nmol/mg of protein, respectively. In summary, the above ESR results are in agreement with the radioactivity data, which showed that atractyloside was able to displace the short-chain (0,2)acyl-[^{3}H]ATR from its binding site, but not the long-chain (7,6)acyl-[^{3}H]ATR (see above). They can be interpreted to mean that, upon addition of atractyloside, the short-chain

TABLE I: Effect of Various Nucleotides and Inhibitors of ADP Transport on the ESR Spectrum of (7,6)Acyl-ATR in Heart Mitochondria.

Added Ligands[a]	Concn (nmol/mg of mitochondrial protein)	Effect[b]
ADP	150	++
ATP	150	++
GDP	150	−
UDP	150	−
ATR	40	++
C-ATR	10	++
BA	25	+
BA + ADP	25 + 25	++

[a] At the concentrations used, the above ligands gave maximal effect. The effect of BA was tested at pH 6.5, obtained by resuspension of the mitochondria in a mannitol–sucrose medium in the presence of 0.02 M Mops, pH 6.5. [b] ++ means strong effect; +, small effect; −, no effect. BA = Bongkrekic acid, C-ATR = carboxyatractyloside.

(0,2)acyl-ATR is released into the water phase and that long-chain acyl-ATRs diffuse away from the ADP carrier into the lipid phase of the membrane.

To further demonstrate the specificity of interaction of acyl-ATRs with the ADP carrier, carboxyatractyloside and bongkrekic acid were used, in addition to atractyloside, to displace the bound (7,6)acyl-ATR. As shown in Table I, carboxyatractyloside was as effective as atractyloside. The releasing effect of bongkrekic acid was increased at pH lower than 7 and by addition of a small amount of ADP or ATP in agreement with the fact that the inhibitory efficiency of bongkrekic acid is enhanced at pH below 7 and by micromolar concentrations of ADP or ATP (Kemp et al., 1970, 1971). In contrast to ADP and ATP, UDP and GDP were totally ineffective; this is in line with the specificity of the ADP carrier for ADP or ATP (Pfaff and Klingenberg, 1968; Winkler et al., 1968; Duée and Vignais, 1969).

(b) Sonic Particles. The following observations indicate that long chain acyl-ATRs do not bind to any protein on the outer face of sonic particles (which corresponds to the matrix face of mitochondria). (1) The ESR spectra of spin-labeled acyl-ATRs incorporated into sonic particles ("inside out" particles) were very close, if not identical, with the spectra of intact ("right-side out") heart mitochondria preincubated with unlabeled atractyloside, and then incubated with spin-labeled acyl-ATRs. (2) When unlabeled atractyloside was added to sonic particles containing spin-labeled long-chain acyl-ATR, there was practically no modification of the spectra.

For comparison with long-chain acyl-ATRs, interaction of long-chain acyl-CoAs with sonic particles has been studied and is briefly reported here. It is known that long-chain acyl-CoAs inhibit ADP transport in whole mitochondria (Pande and Blanchaer, 1971; Shug et al., 1971; Morel et al., 1974). In contrast to long-chain acyl-ATRs, long-chain acyl-CoAs inhibit ADP transport in sonic particles (unpublished). The ESR spectrum of the (10,3)acyl-CoA added to sonic particles (but not that of the (1,14)acyl-CoA) revealed an immobilization which was partially relieved by bongkrekic acid and could therefore be ascribed to an interaction of the (10,3)acyl-CoA with the ADP carrier. The fraction of the (10,3)acyl-CoA which remained immobilized was possibly bound to the pyridine nucleotide transhydrogenase, which is located on the outer

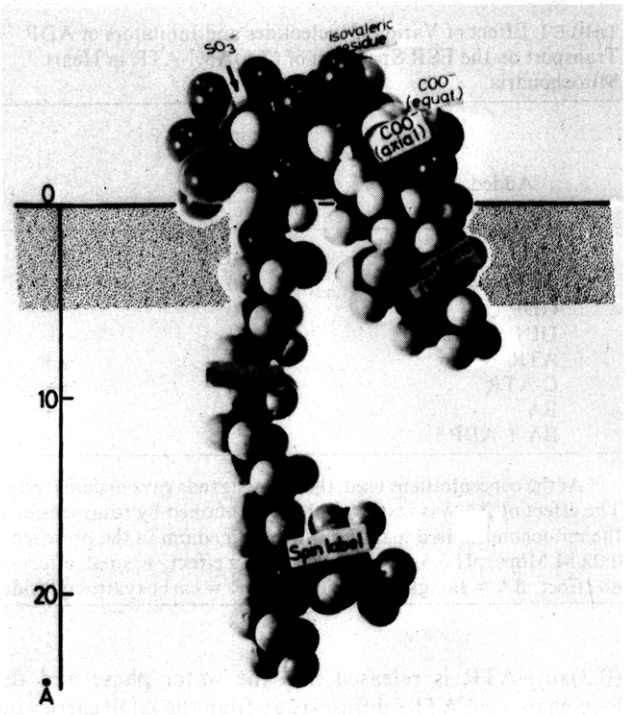


FIGURE 7: Space-filling model of (1,14)acyl-ATR showing the most likely position of the acyl chain and the diterpene residue at the water–lipid interface.

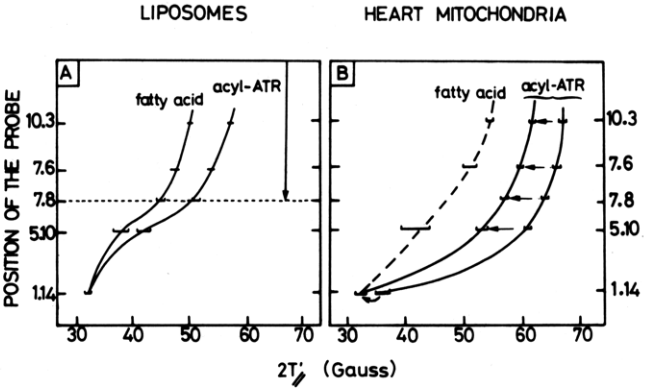


FIGURE 8: Plot of $2T'_{\parallel}$ for different spin labeled fatty acids or acyl-ATRs in liposomes and in beef heart mitochondria. The temperature was 25 °C. The horizontal arrows in B indicate the modification of $2T'_{\parallel}$ values induced by addition of atractyloside at 40 nmol/mg of protein.

side of sonic particles (Rydström et al., 1973; see also Devaux et al., 1975).

In contrast to long-chain acyl-ATRs, the short-chain (0,2)acyl-ATR in sonic particles exhibited an immobilized spectrum which became totally mobile upon addition of atractyloside, carboxy atractyloside, or bongkrekic acid. These ESR data obviously parallel the radioactivity data obtained with the (0,2)acyl-[^{3}H]ATR.

Discussion

(*1*) *Long-Chain Spin-Labeled Acyl-ATR as Model for Determination of the Penetration of a Rigid Hydrophobic Residue in a Lipid Bilayer.* Long-chain acyl-ATR contains two hydrophobic residues: the hydrocarbon chain and the rigid diterpene belonging to the atràctyloside moiety. The addition of a long-chain fatty acid to atractyloside results in a molecule which is less polar than atractyloside. As a consequence, long-chain acyl-ATR binds easily to a lipid bilayer, and it is most likely that both the diterpene and the acyl chain plunge into the lipid bilayer, whereas the polar glucose disulfate remains in the water phase. The diterpene residue is approximately 10–15 Å long and therefore it spans partially the bilayer. The position of the ester linkage should place the fatty acid just beside the diterpene (Figure 7). Because of the length of their acyl chain, palmitic or stearic acid will extend more deeply than the diterpene residue toward the middle of the bilayer. Therefore one may expect to detect the rigid diterpene residue with a probe attached to the fatty acid chain, provided the probe be not too far from the ester linkage. On the other hand, if the probe is near the terminal methyl, its mobility will be characterized by the same order parameter as that found for spin-labeled free fatty acids incorporated in a lipid bilayer.

The ESR results obtained with spin-labeled acyl-ATRs incorporated in liposomes are in agreement with this prediction. In Figure 8A, the value of $2T'_{\parallel}$ is taken as a crude measure of the local disorder (if one assumes a constant polarity of the hydrophobic part of the membrane, the order parameter is then proportional to $2T'_{\parallel}$). As expected, the $2T'_{\parallel}$ value is higher in spin-labeled acyl-ATRs than in the corresponding spin-labeled free fatty acids, when the probe is on one of the first 10 or 12 carbon atoms of the acyl chain starting from the ester bond. A more direct means to ascertain the occurrence of interaction between the diterpene and the acyl chain in long-chain acyl-ATR added to liposomes would be to hydrolyze the glycoside linkage to remove the diterpene, and to compare the ESR spectrum of the newly formed derivative with the spectrum of the original acyl-ATR. However, specific hydrolysis of the glycosidic linkage in acyl-ATR has not yet been achieved.

It is interesting to recall that, in contrast to spin-labeled long-chain acyl-ATRs, spin-labeled long-chain acyl-CoAs incorporated in liposomes show strictly the same spectra as the corresponding spin-labeled free fatty acids (Devaux et al., 1975). Only when the probe was very close to the thioester linkage, can a significant difference in $2T'_{\parallel}$ values of acyl-CoA and fatty acid be detected. The rigid residue linked to the fatty acid in the case of acyl-CoA is the polar CoA moiety. It does not penetrate the lipid bilayer and therefore does not affect the ESR spectra of spin-labeled acyl-CoA.

To summarize, there is strong circumstantial evidence that in acyl-ATR the diterpene interacts with the acyl chain. The interaction of the diterpene residue in acyl-ATR with a spin label placed on the acyl chain is a typical example of intramolecular probing. Such intramolecular probing may be utilized to ascertain the configuration of complex molecules.

(*2*) *Probing of the ADP Carrier by Long and Short Acyl-ATRs. Information on the Asymmetry of the Carrier.* The inhibitory potency of acyl-ATRs on ADP transport was similar to that of atractyloside and it was not modified by a nitroxide radical placed on the acyl chain. These conditions meet the requirement for the spin-labeled acyl-ATR to be used as a probe of the ADP carrier (cf. Devaux et al., 1975).

Incubation of spin-labeled acyl-ATR with whole mitochondria resulted in the immobilization of the probe. The specific binding of spin-labeled acyl-ATR to the ADP carrier was demonstrated by the mobilization of the probe induced by ligands of the ADP carrier. Long-chain acyl-ATRs were displaced from the ADP carrier by ADP, ATR, or other specific ligands, and they moved away from the ADP carrier into the lipid phase of the membrane; only the short-chain (0,2)acyl-ATR moved to the aqueous phase. This behavior was further

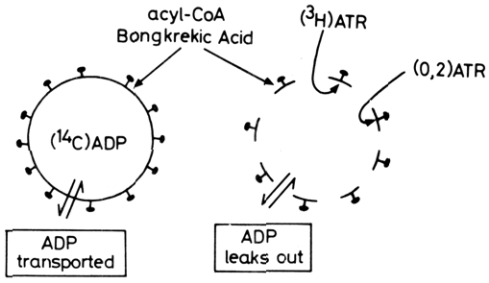


FIGURE 9: Schematic representation of the binding properties of sealed and leaky sonic particles. The "inside out" membrane of the sonic particles is schematized by knobs (F_1-ATPase) facing the medium. Only sealed particles (left) can transport ADP efficiently. On the other hand, only leaky particles (right) can bind ATR or (0,2)acyl-ATR. The binding features of sonic particles are described in Discussion, section 2.

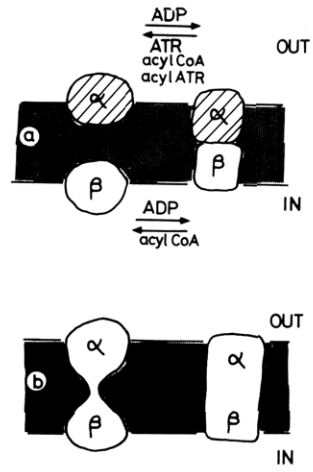


FIGURE 10: Models of ADP-carrier compatible with the ESR results. The left-hand side of the models corresponds to the inhibited configuration (the only explored with specific spin-labeled acyl-ATR); the right-hand side corresponds to the functioning carrier. The transition from the inhibited configuration to the active configuration is induced by ADP, on both sides of the inner membrane. The transition from the active configuration to the inhibited one is induced by the binding of atractyloside (ATR), acyl-ATR, and acyl-CoA on the outer side of the inner membrane and by the binding of acyl-CoA on the inner side. For details, cf. Discussion, section 3.

evidenced by the use of acyl-[^{3}H]ATR, but it was in fact immediately apparent on the ESR spectra. It must, however, be noted that a substantial fraction of the signal corresponding to an immobilized probe still remains after addition of ADP or atractyloside. Indeed the spectra of the displaced spin-labeled acyl-ATRs are not identical with the spectra of the corresponding spin-labeled free fatty acids in mitochondria. This difference is also apparent from Figure 8B, which gives the values of $2T'_{\parallel}$ in heart mitochondria. For the (10,3)-, (7,6)-, (7,8)acyl-ATRs and to some extent for the (5,10)-acyl-ATR, this difference can be explained by intramolecular interaction of the nitroxide with the diterpene residue of the atractyloside moiety (see the first paragraph of this Discussion). However, such a difference should not exist in the case of the (1,14)acyl-ATR since the spectrum of the (1,14)acyl-ATR and that of the (1,14)fatty acid in artificial liposomes are identical. It can therefore be concluded that the displacement of the spin-labeled acyl-ATRs from the ADP carrier is incomplete, in agreement with the fact that the inhibition of ADP transport by acyl-ATRs is a mixed type inhibition. It is noteworthy to recall that the displacement by ADP of spin-labeled acyl-CoAs is complete (Devaux et al., 1975), in agreement with the purely competitive inhibition of ADP transport by acyl-CoAs (Morel et al., 1974). An alternative explanation for the fraction of immobilized signal which remains after addition of ADP is that acyl-ATR binds not only to the ADP carrier, but also to other membrane proteins. The high specificity of the ADP carrier for the atractyloside moiety of acyl-ATR makes this latter explanation unlikely.

Sonic particles were also used to study the interaction of the ADP carrier with spin-labeled acyl-ATRs. These particles were mostly "inside out" as shown under the electron microscope and also by the fact that bongkrekic acid inhibits ADP transport in those particles at pHs higher than 7.5 (unpublished). This latter finding indicates that bongkrekic acid has not to cross the mitochondria membrane in sonic particles to bind to the ADP carrier in contrast to what occurs in intact mitochondria (Lauquin and Vignais, 1976). By using spin-labeled and ^{3}H-labeled acyl-ATR, it was found that the short-chain (0,2)acyl-ATR, but not the spin-labeled long-chain acyl-ATR, interacts specifically with the ADP carrier in sonic particles. The amount of (0,2)acyl-ATR high affinity sites in sonic particles was one-third to one-fifth that found in whole mitochondria per milligram of protein. The same observation holds for [^{3}H]atractyloside. It has been reported (Shertzer and Racker, 1974) and confirmed by us that (1) atractyloside inhibits ADP transport in intact mitochondria, but is totally ineffective when added to sonic particles; and (2) atractyloside inhibits ADP transport when sonic particles have been previously loaded with atractyloside. The same observations apply to the (0,2)acyl-ATR. The above inhibition and binding data are apparently contradictory; however, they can be reconciled, if we admit that a fraction of sonic particles (one-third to one-fifth in the above mentioned experiments), is leaky to atractyloside, (0,2)acyl-ATR, and ADP, but not to long-chain acyl-ATRs. Atractyloside and the (0,2)acyl-ATR would then enter the leaky particles and would bind to their inner face that possesses the high affinity sites; the leaky particles would not obviously transport ADP. On the other hand, the sealed particles can transport ADP by exchange diffusion; being impermeable to atractyloside and to the short chain acyl-ATR, they do not show high affinity sites for these ligands. The behavior of leaky and sealed particles is illustrated in Figure 9.

(3) *Information on the Size of the ADP Carrier and the Mechanism of ADP Transport Deduced from ESR Study.* A previous study with spin-labeled acyl-CoA was interpreted to mean that the ADP carrier in its inhibited configuration, i.e., bound to acyl-CoA, does not extend through the mitochondrial membrane. The results obtained by probing the ADP carrier with spin-labeled acyl-ATR are in agreement with this interpretation since the (1,14)acyl-ATR does not give rise to the strongly "immobilized" spectrum shown by the (5,10)-, (7,8)-, (7,6)-, and (10,3)acyl-ATRs. However, as already discussed, the spectrum of the (1,14)acyl-ATR is immobilized to a greater degree than the spectrum of the (1,14)free fatty acid or the spectrum of the (1,14)acyl-ATR after displacement by atractyloside; it is also immobilized to a greater degree than the spectrum of the (1,14)acyl-CoA in the same mitochondria. On the other hand, a much higher fluidity is found with the (1,14)acyl-ATR added to mitochondria than what would be expected from a spin-labeled fatty acid forced to stay next to a protein extending completely through the membrane, such as cytochrome oxidase (Jost et al., 1973). A plausible explanation is that the atractyloside moiety of the (1,14)acyl-ATR pulls the (1,14)acyl chain toward the aqueous phase, enough to raise the probe in a region of the lipid bilayer where the spin label detects a higher viscosity. Indeed it is well-known by

spin-label studies with fatty acids and phospholipids (McConnell, 1976) that the local viscosity detected by nitroxides is dependent on the exact position of the probe in the lipid bilayer.

In summary, the present spin-label study on acyl-ATR is in agreement with a previous study on spin-labeled acyl-CoA and supports the hypothesis that the inhibited ADP carrier penetrates only partially the mitochondrial membrane. This suggests that the ADP carrier is either a small protein which is able to shuttle between the two faces of the mitochondrial membrane when loaded with ADP or a channel spanning the whole membrane, but very narrow in the middle of the bilayer. Mobile carriers going back and forth across the membrane seem to be thermodynamically unlikely (Singer, 1974). One is then led to consider the association, for the transport process, of two small proteins or subunits, each of them occupying one-half of the bilayer (Figure 10a). One subunit would face the external side of the inner mitochondrial membrane, the other one would face the matrix side. The external subunit (α) would bind ADP, acyl-CoA, and ATR or acyl-ATR, while the internal subunit (β) would bind ADP, acyl-CoA, and bongkrekic acid. The two subunits would be disconnected in the inhibited state or in the absence of substrate; they would be associated only during transport. Such a transport mechanism involving the temporary association of two monomers is well established for gramicidin A (Veatch et al., 1975). Model b in Figure 10 is a modified version of model a. The dimer is now permanent and corresponds to a channel, but the region in the middle of the bilayer is very narrow then inhibition takes place.

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