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AN IN VITRO ESR STUDY OF UNCATALYZED AND RAT LIVER PROTEIN-CATALYZED SPIN-LABELED PHOSPHATIDYLCHOLINE EXCHANGE

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Summary

ESR spectrometry has been used to study fatty acid spin-labeled phosphatidylcholine exchange from single bilayer donor vesicles to various acceptor systems, such as intact or differently treated mitochondria, phospholipid multilamellar vesicles or single bilayer vesicles. This exchange is catalyzed by soluble non-specific rat liver protein, first investigated by Bloj and Zilversmit in 1977 (J. Biol. Chem. 252, 1613—1619). Non-catalyzed phosphatidylcholine exchange has also been studied. Full inhibition of both mechanisms occurs with lipid-depleted acceptor mitochondria, while *N*-ethylmaleimide-treated mitochondria behave as good acceptors during catalyzed exchange but are in no way effective during spontaneous exchange. Non-catalyzed exchange does not take place with phospholipase D-treated mitochondria as acceptors, while the pure catalyzed mechanism is inhibited by 28%. Neither multilamellar nor single bilayer phospholipid vesicles exchange spin-labeled phosphatidylcholine in the absence of protein, the former being a poorer acceptor system than the latter during catalyzed exchange, when this activity is 31 and 80%, respectively, of that of intact mitochondria. The hypothesis is made that the spontaneous mechanism is active among intact natural membranes and could be of some importance in vivo. Furthermore, the biomembrane protein moiety is assumed to be involved in the catalyzed exchange more as a phospholipid spacer than as a binder between the exchange protein and the membrane involved. Phospholipids, on the contrary, appear to be important for both functions.

Introduction

Since indication was first made of the importance of phospholipids as membrane constituents, widespread study has been dedicated to factors affecting the phospholipid composition of natural membranes. Among these factors, great importance has been atrributed to phospholipid exchange, with several studies dealing with this phenomenon. Two main mechanisms have been investigated: spontaneous [1—5] and protein-catalyzed exchange [6–9], with several proteins isolated from various mammalian organs for the latter. Although a great deal of study of these two types of exchange has been made, few data have been obtained regarding their physiological significance.

Our aim was to re-examine the exchange systems in vitro by using an ESR technique affording little or no manipulation of the exchange samples, and testing a variety of artificial and differently treated natural membranes as donor/acceptor systems. Use was made of the partially purified non-specific exchange phospholipid protein from rat liver discovered by Bloj and Zilversmit [10]. Fatty acid spin-labeled phosphatidylcholine exchange activity by this protein was found to depend strictly on the nature of the exchanging membranes. In addition, there is evidence that spontaneous phosphatidylcholine exchange is fully suppressed when artificial or altered natural membranes are used. Discussion is made of the role of both phospholipid and protein components of the exchanging membranes during catalyzed and uncatalyzed phosphatidylcholine exchange, with the hypothesis put forward of the possible physiological significance of spontaneous exchange as compared to the protein-catalyzed phenomenon.

Materials and Methods

Phospholipid exchange protein from rat liver was isolated according to the method of Bloj and Zilversmit [10] up to and including step 3. The second peak fractions were then pooled, concentrated by means of an Amicon ultrafilter and used as such. Column fraction activity was assayed as described in Ref. 10. Protein content determination was carried out by using the method of Lowry et al. [36] as modified for thiol group interference.

1-Acyl-2,12-(4,4-dimethyloxazolidine-*N*-oxyl)stearoylglycerophosphorylcholine (hereafter referred to as spin-labeled phosphatidylcholine) was obtained from egg yolk L-α-lysophosphatidylcholine (Sigma) reacted with 12-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid (Syva, Palo Alto) using the method of Boss et al. [11] and purified by preparative thin-layer chromatography developed with $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v), on 1 mm thick silica gel H plates (Merck).

Single bilayer donor vesicles, obtained according to the method of Brunner et al. [12], contained spin-labeled or [*methyl*-^{14}C]phosphatidylcholine, natural phosphatidylcholine and phosphatidylethanolamine (1 : 1 : 1, molar ratio). In the case of single bilayer acceptor vesicles, the spin- or radioactively labeled phosphatidylcholine was replaced by the natural phospholipid. Reading at 300 nm, only those fractions beyond the absorbance peak were pooled. Lipid phosphorus content was determined using the method of Nakamura [13] and adjusted

to 10—11 mM by means of an Amicon ultrafilter.

Multilamellar vesicles, composed of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (75 : 20 : 5, molar ratio), were prepared according to the method of DiCorleto and Zilversmit [14].

Lipid-depleted mitochondria were obtained according to the method of Fleischer and Fleischer [15].

The phospholipase D incubation medium contained: 1.5 ml of 0.1 mM acetate buffer (pH 5.6), 0.3 ml of 1 M $CaCl_2$, 1 ml enzyme (Boehringer Mannheim) dissolved in acetate buffer (10 mg/ml) and 2 ml mitochondrial suspension (about 80 mg protein). After shaking overnight at room temperature, mitochondria were isolated, washed three times and resuspended at a concentration of about 45 mg/ml protein. The protein content of these particles was assayed by the biuret method. Following thin-layer chromatography and phosphorus content determination, the phospholipid pattern of these mitochondria indicated a greatly lowered phosphatidylcholine and phosphatidylethanolamine content and a higher phosphatidic acid content.

N-Ethylmaleimide mitochondria treatment was carried out by incubating a solution of this reagent at room temperature with 1 ml mitochondrial suspension at a ratio of 50 nmol/mg protein. The mitochondria were then washed three times and resuspended at a concentration of about 45 mg/ml protein.

Protein-mediated exchange assay from radioactively labeled single bilayer donor vesicles to either acceptor mitochondria or multilamellar vesicles was carried out at 37°C as follows: single bilayer ^{14}C-labeled vesicles (150 nmol phospholipid phosphorus) were incubated with 5 mg mitochondrial protein or, alternatively, 8 μmol multilamellar vesicle lipid phosphorus in the presence of 0.45 mg partially purified rat liver exchange protein. Supernatant radioactivity was measured after mitochondria or multilamellar vesicle sedimentation at 8000 or 40 000 $\times g$, respectively.

Exchange assay by EPR measurement was performed using a Jeol JES PE-3X spectrometer. An inital series of experiments was carried out according to the method of Maeda and Ohnishi [16], which involves the use of single bilayer vesicles containing a large amount of spin-labeled phosphatidylcholine and demonstrates the disappearance of the spin-spin exchange band broadening on mixing with natural or artificial membranes. The increase in height of the low-field band due to exchange of the spin-labeled phospholipid was plotted against time. A second series of experiments was performed by direct monitoring of the depth of the low-field band second derivative dip in time. In this case, the spectrometer was operated in the second derivative mode, and the recorder in the Y-T mode. Typical instrumental settings were as follows: 100 kHz superimposed to 80 Hz field modulation frequency for second derivative transformation of the signal; field modulation amplitude, 1.6 G; frequency and intensity of exciting X-band, 9.22 GHz and 40 mW, respectively; amplification, 1.25 $\times$ 100; time constant, 1 s; chart speed, 64 min/360 mm; field set, 3268.2 G (diphenylpicrylhydrazyl EPR marker); 37°C. Each experiment was repeated three times with different exchange protein and donor/acceptor membrane preparations, with excellent agreement. Incubation conditions are given in the legend of the figures.

Results

The ESR experimental technique used in the present work for studying phospholipid exchange is derived essentially from that described by Maeda and Ohnishi [16].

Fig. 1A shows the fatty acid spin-labeled phosphatidylcholine exchange from single bilayer donor vesicles to acceptor mitochondria as followed by repeated scanning of the first derivative spectrum, in the presence of rat liver exchange protein. The increase in spectral intensity clearly shows an active exchange of the spin-labeled phospholipid. Fig. 1B depicts the same experiment as followed by repeated scanning of the second derivative of the low-field absorption line.

The peak amplitude of the negative lobe of the gaussian band second derivative has been determined to be proportional to the band first derivative peak-to-peak amplitude in the ratio $\sqrt{e}/\Delta H_{pp}$ where ΔH_{pp} is the first derivative width [17]. Thus, the higher the ΔH_{pp} value, the lower the second derivative signal. By operating in this way, full advantage is taken of the fact that the low field shoulder in the first derivative spectrum of single bilayer donor vesicles (Fig. 2A) is cancelled in the second derivative spectrum (Fig. 2B). Moreover, in the second derivative mode the spectrometer operates with a higher resolution.

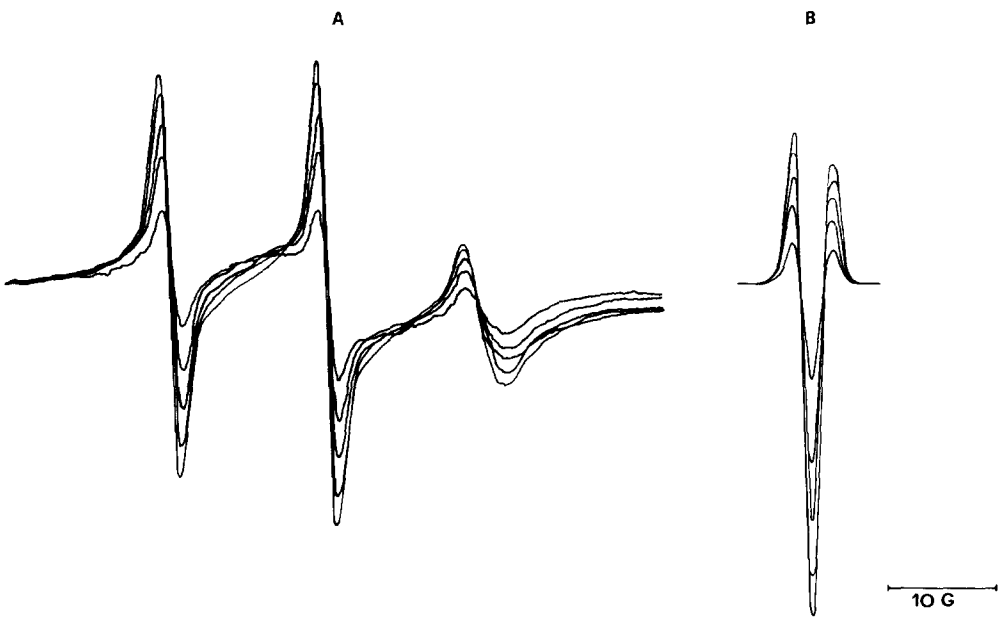

Fig. 1. Exchange of spin-labeled phosphatidylcholine between single bilayer donor vesicles and acceptor mitochondria. The incubation medium (SET buffer, Ref. 10) contained single bilayer vesicles (65 nmol phospholipid phosphorus) (see Materials and Methods), 2.5 mg mitochondrial protein and 0.8 mg partially purified rat liver exchange protein in a final volume of 150 μl. A, repetitive scanning of the first derivative spectrum. B, repetitive scanning of the low-field band second derivative. Spectrometer settings: A, 100 kHz field modulation frequency, 1.6 G field modulation amplitude, 9.22 GHz and 40 mW exciting X-band frequency and intensity, respectively, 0.3 s time constant, 8 min/360 mm scan rate, amplification 3.2 X 100, 8.5 min time interval between each spectrum; B, as in Materials and Methods and same time intervals as in A.

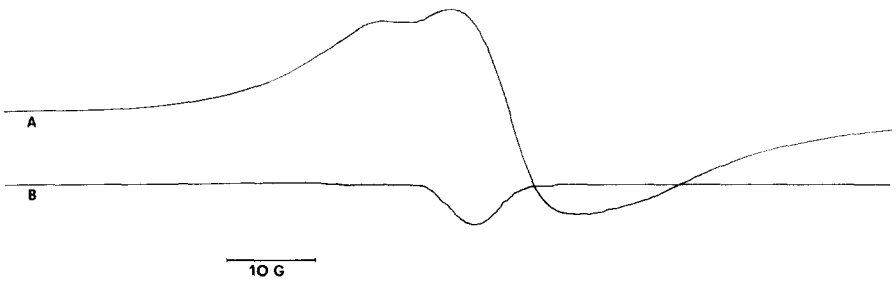

Fig. 2. EPR spectra of single bilayer donor vesicles. A, first derivative; B, second derivative. Spectrometer settings relative to A and B as in Fig. 1.

Fig. 3 shows the results of spin-labeled phosphatidylcholine exchange experiments from single bilayer donor vesicles to both intact or lipid-depleted mitochondria, either with or without exchange protein. Inspection of this figure reveals that an exchange of the spin-labeled phospholipid does take place int he former case even in the absence of protein (Fig. 3B, I). Furthermore, when lipid-depleted mitochondria are used as acceptor membranes in an identical exchange system, the transfer rate is reduced to zero both in the presence (Fig. 3A, III) and in the absence (Fig. 3B, III) of exchange protein. This observation seems to support the idea that true spontaneous exchange also takes place in our system in the absence of soluble protein when intact mitochondria are used as the acceptor system. These data are thus similar to those of other authors [14]. However, we stress that the technique used here proves that the exchange observed is not attributable to adhesion or coprecipitation of single bilayer vesicles with mitochondria. In the present case, exchange activity is restored to a significant extent when normal mitochondria are added to the samples containing lipid-depleted mitochondria, both in the presence (Fig. 3A, II) and in the absence (Fig. 3B, II) of exchange protein. A recovery of 77% for catalyzed and 43% for spontaneous exchange is observed. This is due to added intact mitochondria, indicating that no direct effect is exerted on the exchange protein by lipid-depleted membranes and that exchangeable substrate must in some way be present in the acceptor membranes in order for spontaneous exchange to take place. The fact that a recovery of only 77% is obtained is probably due to the reduced amount of exchange protein, with 25 μl of the medium substituted by an equal amount of intact mitochondrial suspension (see legend to Fig. 3).

The behaviour of both catalyzed and spontaneous exchange activity in the presence of phospholipase D-treated mitochondria as acceptor membranes is shown in Fig. 4A. In this case, the net rate of catalyzed exchange is reduced by 28% in comparison with that obtained when intact mitochondria are used as the acceptor system (compare Fig. 4A, II with Fig. 4A, I). In addition, Fig. 4A, IV shows that the exchange of spin-labeled phosphatidylcholine between single bilayer donor vesicles and phospholipase D-treated mitochondria is almost fully suppressed in the absence of protein. On the other hand, this figure (Fig. 4A, III) confirms the above observations (cf. Fig. 3B, I), according to which the spontaneous single bilayer vesicle-intact mitochondria exchange is in fact taking place.

N-Ethylmaleimide-treated mitochondria have also been used as acceptor membranes, substituting normal mitochondria in exchange experiments. Results with acceptor *N*-ethylmaleimide-treated mitochondria in the presence

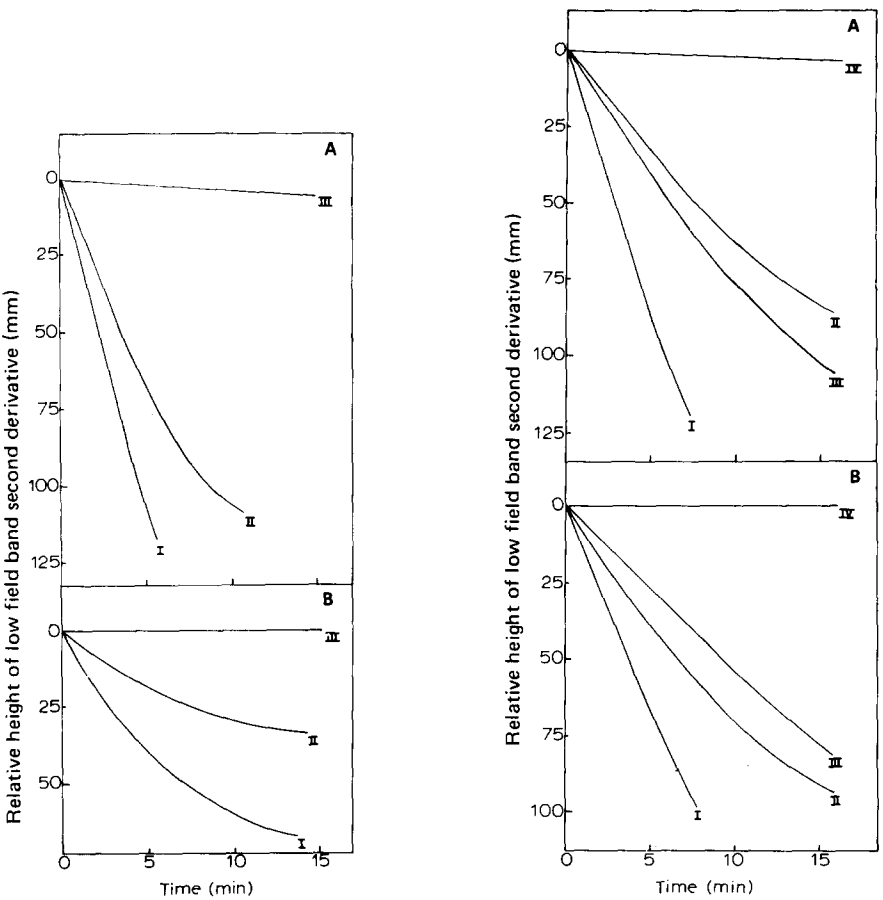

Fig. 3. Exchange of spin-labeled phosphatidylcholine between single bilayer donor vesicles and intact or lipid-depleted mitochondria as followed by the second derivative method. A, 110 nmol vesicular phospholipid phosphorus (see Materials and Methods), 0.4 mg exchange protein, 2.5 mg intact mitochondria (I) or 2.5 mg lipid-depleted mitochondria (III) or 25 μl of the incubated mixture (III) replaced by an equal volume containing 0.7 mg intact mitochondria (II). Final volume, 95 μl. B, I, II and III the same as in A, except that the exchange protein was omitted from the incubation medium being replaced by an equal volume of buffer. Spectrometer settings are given in Materials and Methods. The exchange is proportional to signal intensity increase rate (in mm/min): AI, 21.6; AII, 13.8; AIII, 0.0; BI, 8.2; BII, 3.5; BIII, 0.0.

Fig. 4. Exchange of spin-labeled phosphatidylcholine between single bilayer donor vesicles and acceptor phospholipase D or *N*-ethylmaleimide-treated mitochondria as followed by the second derivative method. A, 55 nmol vesicular phospholipid phosphorus (see Materials and Methods), 0.3 mg exchange protein, 2.5 mg intact mitochondria (I) or 2.5 mg phospholipase D-treated mitochondria (II) or the same as in I (III) and II (IV) except that an equal volume of buffer was used instead of exchange protein; final volume, 95 μl. B, 20 nmol vesicular phospholipid phosphorus, 0.25 mg partially purified exchange protein, 1 mg intact mitochondria (I) or 1 mg *N*-ethylmaleimide-treated mitochondria (II) or the same as in I (III) and II (IV), except that the exchange protein was replaced by an equal volume of buffer; final volume, 95 μl. Spectrometer settings are given in Materials and Methods. The exchange rate is proportional to signal height increase rate (in mm/min): AI, 17.4; AII, 6.7; AIII, 8.1; AIV, 0.0; BI, 13.5; BII, 8.1; BIII, 5.4; BIV, 0.0.

TABLE I

COMPARATIVE EXCHANGE OF [*methyl*-^{14}C]PHOSPHATIDYLCHOLINE BETWEEN SINGLE BILAYER DONOR VESICLES AND EITHER MULTILAMELLAR VESICLES OR MITOCHONDRIA

Final volume, 2.5 ml. Time of incubation, 45 min at 37°C. Figures are the mean of values from five experiments. In each experiment a different protein preparation was used.

Donor [a]	Acceptor	Exchange protein [d]	Total exchanged [^{14}C]phosphatidylcholine (nmol)	Amount of protein-mediated [^{14}C]phosphatidylcholine exchange (nmol)
Single bilayer vesicles	Multilamellar [b] vesicles	absent	0	0
Single bilayer vesicles	Multilamellar vesicles	present	16.8	16.8
Single bilayer vesicles	Mitochondria [c]	absent	49.4	0
Single bilayer vesicles	Mitochondria	present	103.4	54

[a] Single bilayer donor vesicles composed of [*methyl*-^{14}C]phosphatidylcholine/phosphatidylethanolamine (2 : 1, mole ratio) prepared according to the method of Brunner et al. [12], 150 nmol phospholipid phosphorus.

[b] Multilamellar vesicles made up of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (70 : 20 : 5, mole ratio), 8 nmol phospholipid phosphorus.

[c] 5 mg mitochondrial protein.

[d] 0.45 mg partially purified rat liver exchange protein.

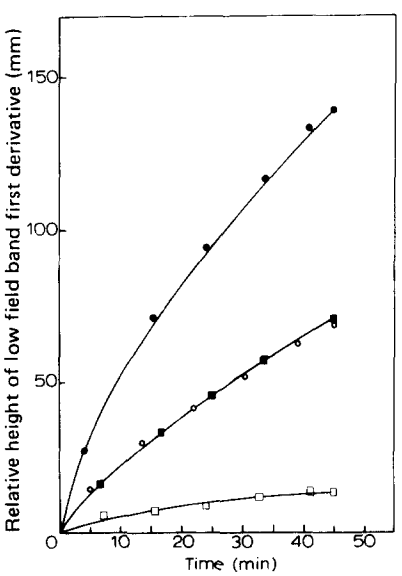

Fig. 5. Exchange of fatty acid spin-labeled phosphatidylcholine between single bilayer donor vesicles and acceptor mitochondria or unlabeled single bilayer vesicles. 2.5 mg acceptor mitochondria incubated [in the presence (●——●) or absence (○——○) of 0.8 mg partially purified rat liver exchange protein] with spin-labeled single bilayer donor vesicles (80 nmol phospholipid phosphorus). 800 nmol phospholipid phosphorus single bilayer acceptor vesicles incubated [in the presence (■——■) or absence (□——□) of 0.8 mg exchange protein] with spin-labeled single bilayer donor vesicles (80 nmol phospholipid phosphorus). Final volume, 150 μl. Spectrometer settings as in Fig. 1.

(II) or absence (IV) of exchange protein, as reported in Fig. 4B, show the net rate of the catalyzed exchange of spin-labeled phosphatidylcholine in the former case to be exactly the same when compared to intact mitochondria. With mitochondria treated in this way as acceptors, uncatalyzed transfer of this phospholipid is completely absent (Fig. 4B, IV). It must be stated that from this figure as well, it can be calculated that uncatalyzed exchange takes place at a significant level with intact mitochondria as acceptors (compare Fig. 4B, III with Fig. 4B, I).

Tests were also made of the ability of multilamellar vesicles to serve as acceptor membranes in comparison with intact mitochondria in catalyzed and uncatalyzed phospholipid exchange. Table I reports the amount of [*methyl*-^{14}C]phosphatidylcholine exchanged after 45 min incubation, both in the presence and in the absence of exchange protein, from single bilayer donor vesicles to multilamellar acceptor vesicles or intact mitochondria. This table shows spontaneous exchange to be completely absent when the acceptor system is an artificial membrane, in agreement with the results of Ref. 14. Moreover, catalyzed exchange appears to be lowered by 69% when compared to intact mitochondria as acceptor membranes. Here again it is confirmed that in the presence of intact mitochondria, using single bilayer vesicles containing [^{14}C]phosphatidylcholine (thus implying a different measurement technique, see Materials and Methods), spontaneous exchange activity is comparable to that shown in Figs. 3 and 4.

Fig. 5 depicts the relative height increase vs. time of the low-field resonance band of the first derivative spectrum following the exchange of spin-labeled phosphatidylcholine between single bilayer vesicles and either intact mitochondria or unlabeled single bilayer vesicles both in the presence and in the absence of exchange protein. The intensity reached after 45 min incubation with single bilayer acceptor vesicles in the presence of exchange protein is lower than with intact mitochondria as the acceptor system. After substracting the final intensity reached by spontaneous exchange in both cases, a reduction is observed of roughly 20% in protein-mediated exchange with single bilayer acceptor vesicles, as compared to intact acceptor mitochondria. With single bilayer vesicles as acceptors, a very low intensity is attained in the absence of protein, indicating the almost complete absence of spontaneous exchange in this case.

Discussion

The data reported in this paper show for the first time that the phospholipid exchange protein, isolated from rat liver cytosol (a non-specific exchange protein), is capable of transferring fatty acyl spin-labeled phosphatidylcholine from single bilayer vesicles to intact mitochondria.

Analysis of Figs. 3—5, which report the results of exchange experiments between single bilayer donor vesicles and acceptor intact mitochondria with and without exchange protein, shows that the exchange is still active even without protein. Keeping in mind the fact that our experimental technique does not involve centrifugation, our data clearly indicate that spontaneous exchange actually does take place in the system under study. This conclusion

is supported by further analysis of Fig. 3, showing data from experiments involving lipid-depleted acceptor mitochondria which still retain their original structure [18]. From this figure it appears that no exchange involving such membranes takes place either in the presence or absence of exchange protein, thus excluding the occurrence of other phenomena such as fusion, absorption or adhesion of single bilayer vesicles to mitochondria [14,19]. An inhibitory effect directly exerted by lipid-depleted mitochondrial membranes on the phospholipid exchange protein is also excluded, since the addition of intact mitochondria to the system containing lipid-depleted mitochondria restores the exchange activity. Of course, the level of restoration depends upon the amount of intact mitochondria added.

Spontaneous exchange of phospholipids has been widely studied, and a complete understanding of this mechanism is beyond the present work. With the sole exception of the data reported by Maeda and Ohnishi [16], this spontaneous phenomenon between single bilayer vesicles has been reported as not occurring [20—22]. The data in Fig. 5, from experiments in which an identical exchange system was tested, confirm that spontaneous exchange between artificial membranes does not occur.

On the other hand, spontaneous exchange between natural membranes has been widely observed, also using spin-labeled phosphatidylcholines analogous or identical to that used by us [23—26]. In addition, a mechanism for spontaneous cholesterol exchange between lipoproteins and erythrocytes has also been proposed, involving local transitory fusion of membranes upon collision that also seems suitable for phospholipids [27]. These considerations, along with our findings, seem to indicate that spontaneous exchange would require at least one natural membrane counterpart in the exchange mechanism. A collision leading to exchange depends upon the chemico-physical conditions of the membranes under contact, as suggested by the observation that cholesterol-depleted membranes do not spontaneously accept cholesterol from lipoproteins [28], whereas intact membrane do [29]. This effect is very similar to that described in Fig. 3, in the presence of lipid-depleted acceptor mitochondria. Thus, the well defined membrane requirement for spontaneous phospholipid exchange occurrence seems to be the integrity of the lipoproteic share. Evidence supporting this proposal is shown in Fig. 4, in which two kinds of pretreated mitochondria were employed, phospholipase D and *N*-ethylmaleimide-treated mitochondria. The aim of the former treatment was to originate a variation in membrane phospholipids; that of the latter to cause variation in membrane proteins. No spontaneous exchange whatsoever occurs in either case. In addition, the data of Table I and Fig. 5 show that in the absence of protein, single bilayer donor vesicles do not exchange spin-labeled phosphatidylcholine with either multilamellar or single bilayer acceptor vesicles. This observation could be interpreted in terms of the definite requirement of membrane proteins in the phospholipid bilayer in order for spontaneous exchange to occur. Furthermore, phospholipid composition effects seem to be excluded since no spontaneous exchange takes place involving multilamellar acceptor vesicles and single bilayer donor vesicles from either asolectin or rat liver mitochondrial phospholipids (unpublished data).

Unlike spontaneous exchange, the most striking overall feature of protein-

catalyzed spin-labeled phosphatidylcholine exchange is its significant residual activity, no matter which type of perturbated membrane is being tested, except in the presence of acceptor lipid-depleted mitochondria, when it is fully suppressed.

Phospholipase D is widely used in order to obtain variations in membrane phospholipids. After treatment of mitochondria with this enzyme, the resulting particles contained a higher amount of phosphatidic acid and behaved as acceptor membranes in a partially inhibitory way on catalyzed exchange (Fig. 4). The effect of phosphatidic acid content in donor or acceptor single bilayer vesicles on catalyzed phospholipid exchange has been widely studied [21,30,31], but at the moment it is still under discussion [32,33]. In our case, inhibition is probably due to the lower content of exchangeable phospholipids of phospholipase D-treated mitochondrial membranes which thus plays a critical role in the exchange rate.

No reduction in the rate of phosphatidylcholine-catalyzed exchange is observed when *N*-ethylmaleimide-treated mitochondria are used as acceptor membranes (Fig. 4B). A similar result has been reported by Kamath and Rubin [34], who alternatively used trichloroacetic acid-treated or boiled acceptor microsomal or donor mitochondria membranes. These data seem to indicate that membrane proteins are not involved in the complex formation between acceptor/donor membrane and the exchange protein. Nevertheless, some effect must be postulated as being exerted by membrane proteins on catalyzed exchange activity, since the data of Table I and Fig. 5 seem to indicate that the absence of proteins in the membrane does render phosphatidylcholine exchange less efficient. Moreover, these data show that catalyzed exchange activity is more affected by the presence of acceptor multilamellar vesicles than by that of single bilayer acceptor vesicles, when compared to intact acceptor mitochondria. DiCorleto and Zilversmit [14] have proposed that single bilayer donor vesicles allow more efficient exchange of phospholipids than donor multilamellar vesicles, probably due to differences in the tightness of packing of these molecules in the two structures. It is most likely that the same effect can be exerted even in our case when these particles perform as the acceptor system. Accordingly, we could postulate that the more efficient exchange observed with intact acceptor mitochondria is attributable to less tightly packed phospholipids in the bilayer when membrane proteins are present, at least for the exchange protein under study.

On the other hand, membrane specificity of exchange proteins cannot be excluded. In fact, it has already been observed that the response of exchange proteins from beef liver and heart towards the same donor system is different [14]. In addition, concerning the exchange activity catalyzed by beef heart protein, no difference has been observed with either phosphatidylcholine/phosphatidylethanolamine/cardiolipin multilamellar vesicle or mitochondria as acceptors [14]. Conversely, the rat liver exchange protein under investigation shows much less activity when intact acceptor mitochondria are replaced by multilamellar vesicles of the same composition.

Thus, some meaningful conclusions can be drawn from the present work. The spontaneous exchange appears to be an important in vivo mechanism, as we have shown that in vitro it is active when at least one intact natural

membrane counterpart is present in the exchange system. This mechanism should be taken into due account when studying the catalyzed exchange since they could act in harmony. Consequently, disregard of the spontaneous mechanism in vitro could be misleading when applying the relative results in vivo.

In addition to phospholipid specificity, membrane specificity must not be underestimated for each isolated exchange protein. In fact, experiments performed in vitro with membranes unlike those in the native environment of the exchange protein could result in conclusions which are not applicable in vivo. In this connection, some criticism has recently been put forward concerning in vitro phospholipid exchange experiments [35].

Finally, we wish to emphasize that the ESR technique, whilst deserving quantitation of its results, seems to be suitable for monitoring both exchange mechanisms, since it does not involve centrifugation or other manipulation of samples, and allows for ready evaluation of spontaneous exchange when studying the catalyzed process.

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