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# Phosphatidylcholine Exchange Protein Catalyzes the Net Transfer of Phosphatidylcholine to Model Membranes†

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ABSTRACT: 2-Stearoyl spin-labeled phosphatidylcholine (PC*) has been introduced into the phosphatidylcholine exchange protein from bovine liver and its electron spin resonance (ESR) spectrum determined. The spin-labeled group in the PC*–exchange protein complex was strongly immobilized. Addition of sodium deoxycholate micelles released PC* from its binding site, producing a mobile signal. This was also observed when micelles of lysophosphatidylcholine and vesicles of phosphatidic acid were added, indicating that the exchange protein can insert its endogenous PC* into interfaces devoid of phosphatidylcholine. ESR spectroscopy was used to measure transfer of PC* from spin-labeled "donor" vesicles to unlabeled "acceptor" vesicles as described by Machida & Ohnishi [Machida, K., & Ohnishi, S. (1978) *Biochim. Biophys. Acta 507*, 156–164]. The donor vesicles consisted of PC* and phosphatidic acid (75:25 mol %) and the acceptor vesicles of phosphatidylethanolamine and phosphatidic acid (81:19 mol %). Addition of exchange protein catalyzed a net tranfer of PC* from donor to acceptor vesicles. This transfer proceeded until the acceptor vesicles contained ~2 mol % of PC*. A spontaneous transfer of PC* was not observed. As for the mode of action, it appears that the exchange protein, after insertion of its endogenous PC* into the acceptor, leaves the interface without a bound phospholipid molecule yet continues to shuttle PC* from donor to acceptor.

The phosphatidylcholine exchange protein from bovine liver is widely used to determine both the size of the PC[1] pool in the outer monolayer of membranes and the rates of transbilayer movement of PC (Zilversmit, 1978; Rothman et al., 1976; Shaw et al., 1979; Van den Besselaar et al., 1978; de Kruijff & Wirtz, 1978). Essential to these studies are the absolute specificity of the protein for PC (Kamp et al., 1977) and its ability to equilibrate PC among "donor" and "acceptor" membranes without changing the size of the PC pools involved (Demel et al., 1973). As for its mode of action, the protein parts with its bound PC molecule upon interaction with a membrane (Kamp et al., 1977). Upon disruption of the protein–membrane complex the free protein contains another PC (Demel et al., 1973). So far, it has not been established whether the protein can leave the interface devoid of PC. This would be required if the protein is involved in net transfer of PC within the cell (Wirtz, 1974).

Recently, ESR spectroscopy has been introduced to monitor continuously the exchange protein catalyzed transfer of 2-acyl spin-labeled (PC*) from PC* vesicles to unlabeled phospholipid vesicles (Machida & Ohnishi, 1978). Evidence was provided that as a putative intermediate in the transfer process a PC*–exchange protein complex was formed characterized by an immobilized spectrum (Devaux et al., 1977; Machida & Ohnishi, 1978). In the present study ESR spectroscopy has

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[1] Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; LPC, lysophosphatidylcholine; PC*, 1-acyl-2-(16-doxylstearoyl)-*sn*-glycero-3-phosphocholine; PE*, 1-acyl-2-(16-doxylstearoyl)-*sn*-glycero-3-phosphoethanolamine; ESR, electron spin resonance; cmc, critical micelle concentration.

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been used to determine the effect of sodium deoxycholate, LPC, PC, and PA on the stability of the PC*–exchange protein complex. In addition, it has been determined whether the protein may catalyze transfer of PC* from PC*-containing vesicles to phospholipid bilayers devoid of PC.

## Experimental Section

*Materials.* PA (sodium salt) and LPC were prepared from egg yolk PC (Makor Chemicals, Jerusalem) as described previously (Kamp et al., 1977). PE was a gift from Dr. B. Schoots and was prepared from egg yolk PC according to the method of Comfurius & Zwaal (1977). Thin-layer chromatography of these phospholipids applied next to known amounts of PC demonstrated that PE, PA, and LPC contained less than 0.1% PC. The lipid spots were visualized by spraying with 20% (v/v) sulfuric acid followed by charring. PC* was prepared from LPC and the anhydride of 16-doxylstearic acid (Hubbel & McConnell, 1971). PE* was derived from PC* by treatment with phospholipase D (Comfurius & Zwaal, 1977). Phosphatidylcholine exchange protein was purified from bovine liver as described (Wirtz et al., 1979). It was stored at −10 °C in 50% (v/v) glycerol at a concentration of 250 μg (9 nmol) of protein per mL. Sodium deoxycholate was purchased from Merck (Darmstadt).

*Preparation of Phospholipid Vesicles.* PC*, PE*, PC, and PE, respectively, were mixed with PA at the desired molar ratio (Wirtz et al., 1979). These mixtures or PA alone was suspended in 10 mM Hepes–sodium hydroxide (pH 7.4)–50 mM sodium chloride at a concentration of 1–10 μmol of phospholipid per mL. The suspensions were sonicated at room temperature under nitrogen with an Ultra-Son Annemasse sonifer until they were clear (~2 times, 5 min) and were used directly. A suspension of LPC was also sonicated briefly to obtain a clear solution.

*Preparation of PC*–Exchange Protein Complex.* Exchange protein [500 μg in 2 mL of 50% (v/v) glycerol] was mixed with vesicles of PC*–PA (98:2 mol %) (1 μmol of phospholipid in 1 mL of Hepes–sodium chloride) and incubated for 90 min at 37 °C by gentle magnetic stirring (Moonen et al., 1979). The PC*–exchange protein complex was separated from vesicles and glycerol by chromatography on a column (18 × 2.1 cm) of Bio-Gel A-0.5m (Bio-Rad Laboratories, Richmond, CA) in 10 mM Hepes–sodium hydroxide–50 mM sodium chloride (pH 7.4). The PC*–PA vesicles eluted in the void volume. The PC*–exchange protein eluted just ahead of the glycerol present in the total volume of the column. Fractions containing the exchange protein were pooled and concentrated in a dialysis bag against dry Ficoll (Pharmacia). For minimization of adsorption, the bag was pretreated with a solution of serum albumin (0.1% w/v). The concentrated protein solution was centrifuged for 15 min at 15000 rpm in a Beckman centrifuge (Model J-21B) to sediment any aggregated protein present. The final protein concentration was ~20 μM (560 μg of protein per mL).

*Electron Spin Resonance Measurements.* Measurements were performed on a Varian E 109 spectrometer in 50-μL quartz cells at 20 °C. The spectrometer was interfaced with a Tektronix 4051 computer for data accumulation. PC*–exchange protein (50–70 μL) was mixed with small aliquots (up to 14 μL) of concentrated solutions of sodium deoxycholate, LPC, and phospholipid vesicles, and the ESR spectra were recorded. In general, because of the relatively low protein concentration, the spectra derive from data accumulation up to 2 h. The protein-mediated transfer between vesicles was determined essentially as described by Machida & Ohnishi (1978). The samples consisted of spin-labeled donor vesicles, unlabeled acceptor vesicles, and exchange protein in a total volume of 60 μL. The transfer of PC* (PE*) was followed with time by measuring the low-field peak-to-peak height of the three-line component (see Figure 2). Scans varied from 2 to 16 min depending on the rate of transfer. As a control donor and acceptor vesicles were mixed and the spectra recorded in the absence of exchange protein.

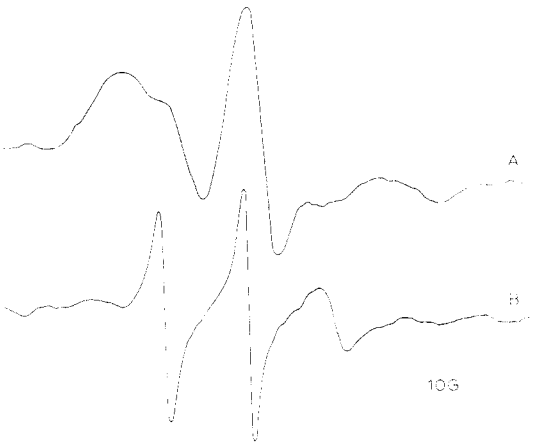


FIGURE 1: ESR spectra of PC*–exchange protein. (A) Sample contained 20 μM protein. Spectrum was accumulated for 80 min at a gain of $1.25 \times 10^5$. (B) Sample contained 17 μM protein in the presence of 0.42% (w/v) sodium deoxycholate. Spectrum was accumulated for 40 min at a gain of $1.25 \times 10^4$.

## Results

*Effect of Detergents and Phospholipid Interfaces.* Figure 1 shows the ESR spectrum (spectrum A) of the PC*–exchange protein complex. The line shape indicates that the nitroxide group on the 2-stearoyl chain is strongly immobilized. In agreement with previous observations (Devaux et al., 1977), the immobilized spectrum was preserved in the presence of 3 mM ascorbate at 0 °C. The failure to reduce the nitroxide group argues in favor of PC* being embedded in the protein, well shielded from the medium.

Addition of sodium deoxycholate to PC*–exchange protein at concentrations below the cmc (0.02, 0.04, and 0.08% w/v, respectively) did not affect the shape of the immobilized spectrum. Above the cmc, addition of deoxycholate (0.14 and 0.42% w/v, respectively) gave rise to the characteristic three-line spectrum (spectrum B, Figure 1). This "mobile" spectrum is due to the incorporation of PC* from the exchange protein into the detergent micelles (Kamp et al., 1975). Dissolution of the micelles by a twofold dilution of the sample containing 0.14% deoxycholate converted instantaneously the mobile spectrum into the immobilized spectrum. This strongly suggested that PC* was reincorporated into the exchange protein. Due to the low PC* concentration after dilution (less than 10 μM) it was not possible to accurately determine the contribution of exchange broadening to the immobilized spectrum. This leaves open the possibility that after dilution some minor amount of PC* may have been involved in label–label association.

The exchange protein does not catalyze the transfer of LPC (Kamp et al., 1977). On the other hand, the protein interacts strongly with LPC micelles (Kamp et al., 1975). Addition of $3.4 \times 10^{-4}$ M LPC to the PC*–exchange protein complex at a lipid/protein mole ratio of 20:1 gave a mobile spectrum identical with spectrum B (Figure 1). In view of the shape of the spectrum, we conclude that the exchange protein, being part of the micellar interface, releases its endogenous PC* into that interface. Similarly, addition of pure PA vesicles to the

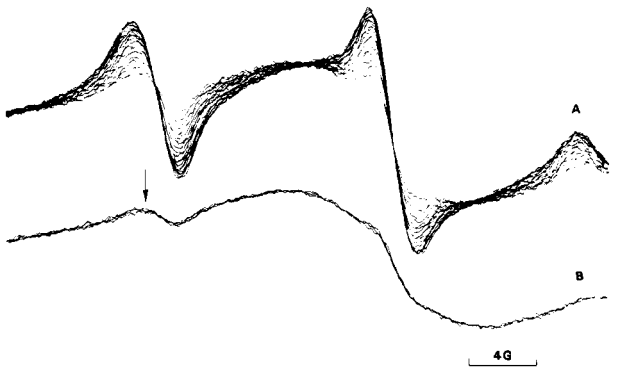


FIGURE 2: Change in the ESR spectrum of PC*-containing donor vesicles after addition of acceptor vesicles and exchange protein. (A) Donor vesicles (6.3 nmol of PC*–PA, mole ratio of 75:25) were mixed with acceptor vesicles (57 nmol of PE–PA, mole ratio of 81:19) and exchange protein (12 $\mu$g) in a total volume of 60 $\mu$L of Hepes–sodium chloride (pH 7.4) and incubated at room temperature. (B) Without exchange protein. Scan rates of 8 min were recorded at a gain of $4 \times 10^4$.

complex at a lipid/protein mole ratio of 75:1 produced the mobile spectrum. This implies that the exchange protein has released its PC* into a bilayer interface that is devoid of PC. Interaction of the PC*–exchange protein with PC-containing vesicles produced the same mobile spectrum as a result of exchange.

*Net Transfer of PC**. Release of PC* into interfaces of LPC and PA suggested that the exchange protein may catalyze a net transfer of PC*. This was examined by measuring the transfer from donor vesicles of PC* and PA (75:25 mol %) to acceptor vesicles of PE and PA (81:19 mol %). In the absence of exchange protein, mixing of donor and acceptor vesicles gave the ESR spectrum shown in Figure 2 (spectrum B). This spectrum resembled that of the donor vesicles alone except for a mobile component (see arrow) of which the peak height did not vary with time. This mobile component was virtually eliminated by addition of bovine serum albumin (final concentration of 2% w/v). This suggests that the donor vesicles contained a small quantity (less than 1%) of spin-labeled fatty acid that redistributed very rapidly between the two types of vesicles. Spin-labeled fatty acid may have been generated during sonication.

Addition of exchange protein to the mixture of donor and acceptor vesicles gave rise to the time-dependent appearance of a three-line spectrum, indicating that PC* was transferred from donor to acceptor (Figure 2, spectrum A). Under the conditions of incubation (see legend to Figure 2A) the spectrum attained its final shape after ~2 h, at which time the level of PC* in the acceptor vesicles had reached a maximum. In the reverse experiment where PC* in the donor vesicles was replaced for PE* and PE in the acceptor vesicles was replaced for PC, the exchange protein did not induce any spectral change, confirming that the protein does not transfer PE* (Kamp et al., 1977). These two complementary experiments demonstrate that the exchange protein incorporates PC* into the PE-containing acceptor interface without a transfer of PE in the opposite direction; that is, the protein catalyzes a net transfer.

Spectra as shown in Figure 2 have been recorded at three concentrations of exchange protein (6, 12, and 24 $\mu$g of protein, respectively), and the increase of the low-field peak-to-peak height has been plotted against time (Figure 3A). Under the conditions of incubation the contribution of the exchange-broadened spectrum to the height of the low-field peak is negligible. The low-field peak, therefore, is a measure of the

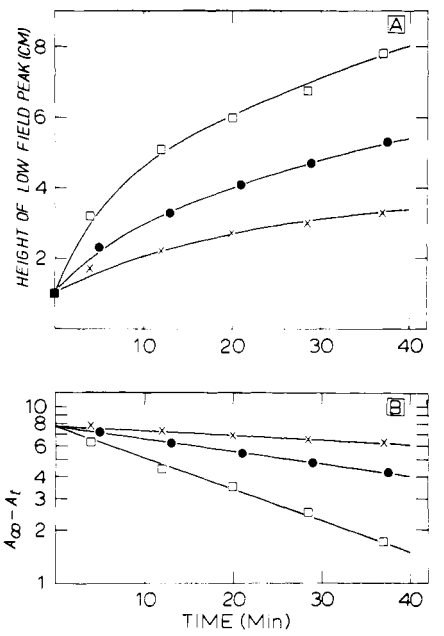


FIGURE 3: Incorporation of PC* into acceptor vesicles as a function of time and exchange protein. For incubation conditions, see the legend to Figure 2. (A) Incorporation of PC* is measured by monitoring the increase of the low-field peak from spectra as depicted in Figure 2. (B) Semilogarithmic plot of $A_\infty - A_t$ vs. time, where $A_\infty$ and $A_t$ are the heights of the low-field peak at infinity and time $t$, respectively. (×) 6 $\mu$g of protein; (●) 12 $\mu$g of protein; (□) 24 $\mu$g of protein.

extent at which PC* is incorporated into the acceptor vesicles. This is corroborated by the plots of Figure 3A which indicate that the initial rate of PC* transfer increased proportionally with the amount of exchange protein. In addition, one can derive from Figure 3A that transfer of PC* proceeds according to an exponential function. This is borne out by the linearity of the semilogarithmic plot of $A_\infty - A_t$ against time where $A_\infty$ and $A_t$ are the low-field peaks at infinity and time $t$, respectively, corrected for the blank.

By integrating the whole spectrum at maximal PC* transfer and taking into consideration the contribution of the exchange-broadened spectrum (spectrum A, Figure 2), we estimated that the acceptor vesicles contained 20 ± 5% of the donor PC*. This is ~2% of the total acceptor phospholipid since the incubation mixture contained 4.7 nmol of donor PC* and 57 nmol of acceptor phospholipid. Spectra were also recorded under conditions where the acceptor phospholipid concentration was varied at a constant amount of 4.7 nmol of donor PC*. The heights of the low-field peak of these spectra at maximal transfer are presented in Figure 4. At 57 nmol of acceptor phospholipid, incorporation of 1 nmol of donor PC* gives rise to a low-field peak of 12 cm. At lower acceptor phospholipid concentration the height of the low-field peak becomes considerably less, indicating that the acceptor vesicles have become limiting to the level of maximal incorporation of donor PC*. Due to the relatively large error in the calculation, the actual amounts of PC* transferred could only be roughly estimated but are in the range of 2% of the acceptor phospholipid. On the other hand, if the acceptor phospholipid concentration is raised to 114 nmol, the height of the low-field peak increases slightly, indicating that transfer of PC* is still on the order of 20% of the total donor PC*. This suggests that under these conditions the donor vesicles have become limiting (see Discussion). Additional support for this conclusion derives from the following experiment. Incubation of 12 $\mu$g of exchange protein with 6.3 nmol of donor PC*–PA (75:25 mol %) and 114 nmol of acceptor PE–PA (81:19 mol

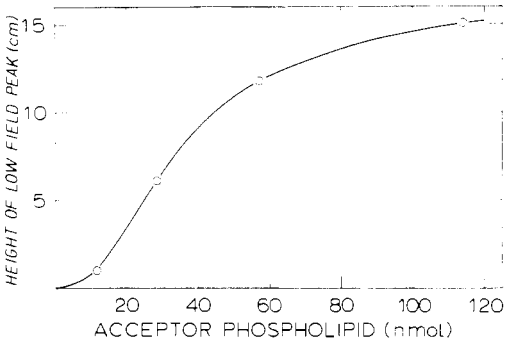


FIGURE 4: Height of the low-field peak as a function of acceptor phospholipid concentration. Donor vesicles (6.3 nmol of PC*–PA, mole ratio of 75:25) were incubated with various amounts of acceptor vesicles and 12 μg of exchange protein. For further incubation conditions, see the legend to Figure 2. The height of the low-field peak was measured after PC* had equilibrated between donor and acceptor. Spectra were recorded at a gain of $4 \times 10^4$.

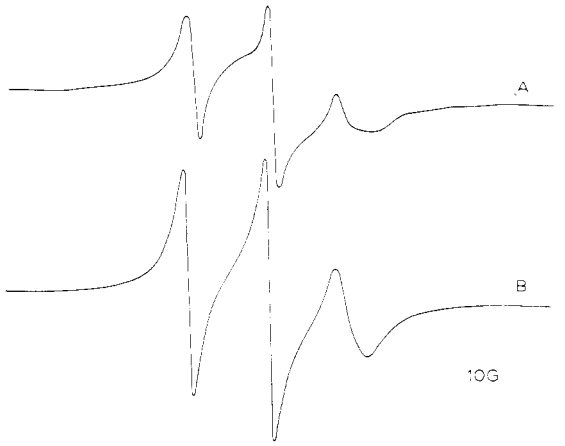


FIGURE 5: ESR spectra of a mixture of donor vesicles and acceptor vesicles of varying phospholipid composition and exchange protein, at equilibrium. Donor vesicles (6.3 nmol of PC*–PA, mole ratio of 75:25) were mixed with 12 μg of exchange protein and incubated at 20 °C with acceptor vesicles (114 nmol of PE–PA, mole ratio of 81:19) for 2 h (spectrum A) or with acceptor vesicles (114 nmol of PC–PA, mole ratio of 82:18) for 20 min. Spectra were recorded at a gain of $2.5 \times 10^4$.

%) produces at equilibrium spectrum A of Figure 5. If under similar conditions PE in the acceptor vesicles is replaced for PC, incorporation of PC* into the acceptor will proceed by protein-mediated exchange, giving rise to the equilibrium spectrum B of Figure 5. It is evident from spectra A and B that under the conditions of exchange as compared to net transfer, incorporation of PC* into the acceptor vesicles has been more extensive. This agrees with the fact that by exchange ~60% of the donor PC*, i.e., the outer membrane pool, is involved and by net transfer ~20%. It is of interest that protein-mediated exchange produces spectrum B within 20 min whereas it takes 2 h of net transfer to produce spectrum A.

## Discussion

Phosphatidylcholine exchange protein has a binding site for PC, which, in part, is formed by the extremely hydrophobic hexapeptide -Val-Phe-Met-Tyr-Tyr-Phe- (Moonen et al., 1979). Occupation of this site by 2-stearoyl spin-labeled PC (PC*) gives the ESR spectrum characteristic for a strongly immobilized probe, confirming previous observations (Devaux et al., 1977; Machida & Ohnishi, 1978). Addition of sodium deoxycholate micelles to the PC*–exchange protein complex gives the mobile spectrum (Figure 1). This reflects the formation of mixed PC*–deoxycholate micelles, as has been shown in a previous study (Kamp et al., 1975). Elimination of the micelles by dilution allows PC* to reenter its hydrophobic crevice, as reflected by the recurrence of the immobilized spectrum. This implies that the binding site remains accessible for PC in the presence of relatively high monomer concentrations of detergent (e.g., 0.07%). At present we do not know whether deoxycholate interacts with the hydrophobic site in a fashion comparable to its interaction with hydrophobic proteins (Helenius & Simons, 1971).

Addition of PC*–exchange protein to receptor-rich membranes from *Torpedo marmorata* released PC* into these membranes (Rousselet et al., 1979). The present study shows that the exchange protein released its endogenous PC* also into interfaces made of LPC or PA. In both instances the mole ratio of PC*–exchange protein to LPC (1:20) and to PA (1:75) was such that the release of PC* could not be explained by exchange with PC present as a contaminant (less than 0.1%). This implies that the endogenous PC on the protein need not remain with the binding site when the protein forms a complex with interfaces devoid of PC. This property is one of the prerequisites for the exchange protein to catalyze net transfer of PC. In a similar study, Machida & Ohnishi (1978) added vesicles of pure PS to the PC*–exchange protein complex in a molar ratio of 160:1 and 600:1. In spite of strong binding of the protein to the vesicles, only a partial release of PC* was observed. This release was more pronounced at the higher PS concentration. These observations differ markedly from ours with PA and suggest that with the exchange protein bound to the vesicle interface PC* has a greater degree of freedom in a bilayer of PA than of PS. This could mean that in the exchange protein–interface complex PA competes with PC* for the lipid binding site more effectively than PS does.

ESR spectroscopy has proven itself to be a very sensitive technique to measure transfer of PC* from PC* donor vesicles to nonspin-labeled acceptor vesicles (Maeda & Ohnishi, 1974; Machida & Ohnishi, 1978). Because PC* transferred to the acceptor produces a completely different spectrum from PC* in the donor, protein-mediated transfer of PC* can be followed without physically separating the vesicles. This provides a large advantage over the conventional radioactive assays [for a review, see Zilversmit & Hughes (1976)], particularly under conditions where one deals with relatively low levels of transfer. In the present study, the donor vesicles consisted of PC*–PA (75:25 mol %) and the acceptor vesicles of PE–PA (81:19 mol %). Spontaneous transfer of PC* and/or fusion of donor and acceptor was negligible (20 °C, 2–3 h of incubation) probably due to the high negative surface charge on these vesicles. Spontaneous exchange of phospholipids has been detected between vesicles consisting of 82 mol % PE and 18 mol % acidic phospholipids (Duckwitz-Peterlein et al., 1977). Although these vesicles resemble those of the present study, exchange was extremely slow with half-times of 30–80 h at 45 °C.

Addition of exchange protein to the mixture of donor and acceptor vesicles catalyzed a net transfer of PC* to the PE–PA vesicles (Figure 2). Incorporation leveled off when PC* consituted ~2% of the acceptor phospholipid pool. Because the transfer of PC* fits an exponential function, it appears that an initial net incorporation of PC* is gradually replaced by exchange going toward equilibrium concentration. At present little is known about the physical–chemical parameters of the PE–PA interface (Stollery & Vail, 1977). It seems, however, likely that the phospholipid composition of the acceptor in conjunction with the affinity of the protein for PC* exposed at the interface dictates the equilibrium concentration. In a

previous study (Kamp et al., 1977) it was demonstrated that the PC–exchange protein did not release its endogenous PC into vesicles of pure PE. Apparently, the presence of PA in an interface of predominantly PE facilitates this release process. Net transfer implies that the donor vesicles become depleted of PC. By increasing the concentration of the acceptor vesicles relative to the donor vesicles, we observed that protein-mediated net transfer stopped at a depletion of ~20% (Figure 4). On consideration of the size distribution of sonicated PC vesicles (Kornberg & McConnell, 1971; Hauser & Irons, 1972), a depletion of 20% seems reasonable if we assume that mainly the larger donor vesicles are involved, becoming smaller in the process. Protein-mediated net transfer of PC* to PE–PA acceptor vesicles proceeded at a much lower rate than the protein-mediated exchange of PC* with PC–PA acceptor vesicles (see also Figure 5). This difference in rate may reflect a relatively low affinity of the exchange protein for the PE–PA vesicles. On the other hand, the protein-mediated net removal of PC* from the donor vesicles may be a rate-limiting step.

Net transfer of PC* implies that the exchange protein leaves the interface devoid of PC and as a delipidated protein extracts another PC* molecule from the donor. In a previous study (Kamp et al., 1975) it was already demonstrated that the exchange protein delipidated by detergents kept its transfer activity. In summary, the present study strongly suggests that as for the mode of action of the exchange protein, insertion of PC into an interface and extraction of PC from an interface are independent events.

Except for the phosphatidylcholine exchange protein, other exchange proteins exist that may catalyze a net transfer of phospholipids. The phosphatidylinositol (PI) exchange protein from bovine brain and heart transfers PI from a donor membrane to PC-containing vesicles deficient in PI (Harvey et al., 1974; Demel et al., 1977; DiCorleto et al., 1979; Zborowski, 1979). In these experiments, however, it was evident that due to a dual specificity the protein exchanged PI for PC and did not catalyze an actual net transfer. This exchange mechanism may also explain the stimulatory effect of a pH 5.1 supernatant from rat liver on the transfer of PI from pure PI to pure PC vesicles (Barsukov et al., 1975). It is of interest that a partially purified exchange protein from bovine heart has been shown to catalyze a net transfer of PC from PC vesicles to a reconstituted membrane consisting of PE and cardiolipin (80:20 mol %) and mitochondrial hydrophobic protein (Kagawa et al., 1973). Although incorporation of PC by exchange was not rigorously excluded, it may well be that our present findings apply to this study.

It has been proposed that phospholipid exchange proteins are involved in membrane biogenesis and renewal of membrane phospholipids (Wirtz, 1974; Kader, 1977). Protein-mediated net transfer of phospholipids would be required in these phenomena. We as yet do not know whether the factors that regulate the transfer activity in vitro are relevant to intracellular phospholipid transport. Recent studies, however, do suggest that in vivo there may be a relationship between phospholipid exchange activity and the ability of the cell to synthesize phospholipids or generate membranes (Engle et al., 1978; Brophy & Aitken, 1979).

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