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TRANSFER OF PHOSPHOLIPIDS BETWEEN MEMBRANES

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I. Introduction 95
II. Exchange of phospholipids and the serum lipoproteins 96
III. Exchange of phospholipids between membrane structures 98
IV. Exchange of phospholipids and the 150 000 × *g* supernatant 100
V. Some properties of the pH 5.1 supernant fraction 103
VI. Phospholipid-exchange proteins 105
VII. Phosphatidylcholine-exchange protein 107
  A. Properties 107
  B. Mode of action 110
VIII. Possible role of the phospholipid-exchange proteins 111
Acknowledgements 114
References 115

I. INTRODUCTION

A variety of techniques, both biochemical and physical, have shed light upon the structure of biological membranes. A consensus of opinion has developed by which the membrane is thought to consist of a phospholipid bilayer interspersed with proteins. Excellent reviews on this subject have recently been published [1–3]. It is interesting to note that the current membrane model accentuates the fluidity of the membrane. Phospholipids undergo a rapid lateral diffusion within the two monolayers of the bilayer [4–7]. Membrane proteins are also thought to diffuse freely in the lipid matrix [8–11]. Which phospholipids and proteins in the membrane are in this state of motion, and how the movement of phospholipids and proteins is interrelated, are the subjects of intensive research [12–17].

It is not known in what manner the two phospholipid monolayers of the bilayer are related. At this stage it is valid to assume that the two monolayers are discrete entities, with little or no mixing of phospholipids between the two halves of the

bilayer. This is borne out by the evidence that the phospholipids are asymmetrically distributed over the inside and outside of the erythrocyte membrane [3,18–20]. Exchange of spin-labelled phosphatidylcholine between the inside and outside of an artificial membrane such as that of liposomes ("flip-flop") was demonstrated to be slow with a half-time of 6.5 h at 30 °C [21]. Additional studies, however, on the rate of flip-flop of spin-labelled phosphatidylcholine in natural membranes such as those of erythrocytes, *Acholeplasma laidlawii* and the electroplax of *Electrophorus electricus* gave half-times in the order of minutes [22,23]. If other membrane phospholipids also participate in this flip-flop mechanism, then the asymmetric distribution of phospholipids observed, for example, in the erythrocyte membrane may reflect an equilibrium distribution rather than being a direct consequence of biogenetic events.

In this review another aspect of phospholipid movement will be discussed which should be considered to appreciate the full role of phospholipids in a biological membrane. This is the movement, or exchange, of intact phospholipid molecules between membrane structures. Recently a review on this subject has been published in which the author discussed the exchange of phospholipids particularly with respect to the total phospholipid metabolism of the cell [24]. The present review will summarize the evidence for phospholipid exchange and will discuss the possible implications this process may have for membrane biogenesis.

II. EXCHANGE OF PHOSPHOLIPIDS AND THE SERUM LIPOPROTEINS

In general, the studies on phospholipid exchange involve two distinct structures one of which initially contains labelled phospholipid molecules. Exchange of phospholipids is then defined as the redistribution of label between the two structures while the phospholipid content of each structure remains unaltered in the process.

The earliest observations on the exchange of phospholipids between discrete structures were made on serum lipoproteins. It was the introduction of ultracentrifugal techniques to separate various classes of lipoproteins which led to the demonstration that phospholipids exchanged between the very low density, low density and high density lipoproteins [25–27]. It was suggested that this exchange was due to the formation of collision complexes between the lipoproteins [28]. Provided phospholipids could diffuse in this complex, dissociation of the complex would be reflected in an exchange of phospholipids. However, as has been recently shown, investigation of this particular exchange process is complicated by the fact that some of the lipoprotein subunits such as lipoprotein apoprotein–glutamic acid and lipoprotein apoprotein–alanine also exchange [29]. It is conceivable, therefore, that phospholipid–lipoprotein apoprotein subunits exchange between the lipoproteins in which process the protein functions as a carrier of the phospholipid [30,31].

Although exchange of phospholipids between erythrocytes and plasma has been observed by many investigators [32–36], it is still not absolutely clear what this process actually entails. Is it simply an exchange of phospholipids, or is this ex-

change linked to some other metabolic processes involving phospholipids of both erythrocytes and plasma? In this regard the effects of lecithin:cholesterol acyltransferase activity of plasma [37] and the acylation activity of erythrocytes [38,39] on the exchange should be considered.

Actual rates of exchange were determined by Reed [35]. It was found in vitro for plasma and erythrocytes of both man and dog that phosphatidylcholine had a 12-h fractional turnover of 13% and sphingomyelin of 14%. Phosphatidylserine and phosphatidylethanolamine of the erythrocyte were not involved in this exchange. This may reflect the absence of these phospholipids in plasma, or on the other hand, it would agree with the concept that these phospholipids are situated on the inside of the erythrocyte membrane [18,20].

In a few instances erythrocytes have been incubated with isolated ^{32}P-labelled plasma lipoproteins. Under these conditions Soula et al. [36] observed a very rapid, time-independent transfer of label to rabbit erythrocytes. Autoradiograms of the chromatographed erythrocyte phospholipids demonstrated that the label incorporated in the erythrocytes was mainly present in lysophosphatidylcholine. From labelling studies in vivo Soula et al. [40] concluded that exchange of phospholipids between chicken erythrocytes and plasma was very slow and quantitatively different from mammalian systems. Evidence has been given that phospholipids can also exchange between serum lipoproteins and subcellular membrane fractions such as rat liver microsomes [41], rat liver mitochondria [42] and plasma membranes from squirrel monkey liver [43].

It is presumed that the exchange is the result of collisions between lipoproteins and membrane structures. If so, the collisions must be rather ineffective since the exchange is slow compared to the phospholipid exchange between subcellular particles discussed in Section IV. At this point it is uncertain what physiological purpose is served by the exchange involving serum lipoproteins. Whether the exchange affects the phospholipid composition of the membranes involved has not been ascertained. It is interesting to note that the choline-containing phospholipids are located on the outside of the human erythrocyte membrane [18,20] and that it is these phospholipids which are predominantly present in the plasma [44]. This raises the important question whether the asymmetric distribution of phospholipids in the erythrocyte membrane is intrinsic to its biogenesis or could possibly be effected by secondary processes such as the exchange discussed above. The same question pertains to the phospholipid composition of the plasma membrane, which is generally shown to have a much higher sphingomyelin content than the intracellular membranes [45,46]. It would be of interest to know whether this phospholipid is concentrated on that side of the membrane which is in direct contact with the plasma.

It has been shown that phospholipids exchange between cultured chick embryo fibroblasts and the serum in the growth medium [47]. This has been confirmed for rapidly dividing human prostatic epithelial cells in tissue culture [43]. By analogy it is possible that in vivo the phospholipid pools of different tissues are in a dynamic state of equilibrium mediated to some extent by serum lipoproteins. In this concept

plasma membranes would be the initial sites of exchange. LeKim et al. [48] have shown that upon intravenous administration of doubly labelled phosphatidylcholine to rats, the complete phospholipid molecule was incorporated into a large number of tissues. It was concluded that this incorporation reflected a continuous exchange of phospholipids between serum lipoproteins and these tissues. Upon cell fractionation it was found that the label was present in intracellular membrane structures such as mitochondria and microsomes. Whether label was incorporated into plasma membranes was not determined. A mechanism by which phospholipids may be distributed throughout the cell will be discussed in the following sections.

III. EXCHANGE OF PHOSPHOLIPIDS BETWEEN MEMBRANE STRUCTURES

Phospholipids normally found in biological systems, have an exceedingly low critical micelle concentration. For example, dipalmitoylphosphatidylcholine has a critical micelle concentration of $4.6 \cdot 10^{-10} \pm 0.5 \cdot 10^{-10}$ M at 20 °C [49]. This implies that transfer of such phospholipid molecules between two interfaces separated by an aqueous phase should be negligible. In fact, it was shown by Kornberg and McConnell [21] that on incubation of phosphatidylcholine liposomes containing 15 mole % spin-labelled phosphatidylcholine with pure phosphatidylcholine liposomes, the spin label was not redistributed between the two classes of vesicles. This has been confirmed by Ehnholm and Zilversmit [50] who measured the exchange of phosphatidylcholine between liposomes sensitized with Forssman antigen and non-sensitized liposomes. Separation of the liposomes by addition of antiserum γ-globulin showed little or no exchange even after a 12-h incubation at 37 °C. These studies also indicated that, even if collisions were taking place between the liposomes, these collisions did not bring about phospholipid exchange. This may reflect the stability of the vesicles both in terms of packing of phosphatidylcholine molecules in the bilayer and in terms of the stabilizing force exerted by the electrical double layer. On the other hand, destabilizing the liposomal structures with, for example, lysophosphatidylcholine results in fusion [51].

In the last few years, the exchange of phospholipids between natural membranes, particularly mitochondria and microsomes from rat liver, has been a subject of much research. This interest stemmed originally from the observation that in vivo rat liver mitochondrial phospholipids became very rapidly labelled after administration of a precursor isotope [52,53], although synthesis of phospholipids was thought to occur predominantly in the endoplasmic reticulum [54–56]. Radioautographic studies of Stein and Stein [57] using [^{3}H]choline and pulse-chase experiments of Jungalwala and Dawson [58] using labelled phospholipid precursors in isolated hepatic cells strongly suggested that newly synthesized phospholipids were rapidly redistributed between the endoplasmic reticulum and mitochondria by exchange. Wirtz and Zilversmit [59] arrived at a similar conclusion by establishing that the specific radioactivity of the mitochondrial phospholipids changed in parallel with that of the

TABLE I

EFFECT OF PHENOBARBITAL AND CARBON TETRACHLORIDE ON THE INCORPORATION OF $^{32}P_i$ INTO MICROSOMAL AND MITOCHONDRIAL PHOSPHOLIPIDS

Microsomes and mitochondria were isolated 1 h after $^{32}P_i$ administration. Numbers preceded by ± are standard deviations. For further experimental details, see ref. 59.

	Phenobarbital (5)*	Control (4)*	Carbon tetrachloride (3)*	Control (3)*
Specific activity (counts/min per μg phospholipid-*P*)				
Microsomal				
Phosphatidylcholine	337 ± 48**	578 ± 57	332 ± 24	369 ± 52
Phosphatidylethanolamine	861 ± 49	1107 ± 55	1304 ± 93	613 ± 138
Mitochondrial				
Phosphatidylcholine	197 ± 35	302 ± 42	167 ± 21	190 ± 42
Phosphatidylethanolamine	320 ± 21	398 ± 15	348 ± 22	162 ± 43
Ratio specific activity of mitochondria to that of microsomes				
Phosphatidylcholine	0.58 ± 0.03	0.52 ± 0.03	0.50 ± 0.03	0.51 ± 0.05
Phosphatidylethanolamine	0.37 ± 0.02	0.36 ± 0.03	0.27 ± 0.01	0.26 ± 0.01

* Number of animals in parentheses.
** All differences between experimental and control values are significant at the 5% level.

microsomal phospholipids following treatment of rats with phenobarbital or carbon tetrachloride. These agents are known to interfere, among other things, with the phospholipid metabolism of, specifically, the endoplasmic reticulum [60–62]. It is shown in Table I that phenobarbital treatment lowered proportionately the specific activities of microsomal and mitochondrial phosphatidylcholine and phosphatidylethanolamine while carbon tetrachloride treatment increased proportionately the specific activity of microsomal and mitochonderial phosphatidylethanolamine.

How is the intracellular transfer of phospholipids between the endoplasmic reticulum and mitochondria brought about? From evidence based on electron microscopy it has been argued that the outer mitochondrial membrane and the endoplasmic reticulum form a continuum [63]. Lateral diffusion of phospholipids within this continuum would explain the observed redistribution of label. While this interpretation cannot easily be refuted, additional techniques should be used to confirm the existence of such a continuum. Another possible explanation could be that through movement within the cell mitochondria collide with the endoplasmic reticulum exchanging their phospholipids in the process. Movement of mitochondria in situ has been demonstrated cinematographically by Frederic [64] in tissue culture cells. A third possibility, and the principal subject of this review, is the role of the phospholipid exchange proteins in shuttling phospholipids back and forth between the various subcellular organelles.

Wirtz and Zilversmit [42,65], McMurray and Dawson [55] and Akiyama and

Sakagami [66] investigated in vitro the exchange of phospholipids between mitochondria and mirosomes from rat liver. It was observed that the exchange was little, but was greatly stimulated upon addition of a 105 000 × *g* supernatant. It cannot be excluded that the exchange observed in the absence of this fraction, was due to some supernatant proteins still present in the subcellular particles [42]. By and large it is doubtful whether exchange of phospholipids between mitochondria and microsomes can occur without participation of this soluble protein fraction. In this respect it should be mentioned that both mitochondria and microsomes are stabilized by a negative surface charge [67]. Charge repulsion may prevent an effective collision in vitro between these particles and as such preclude exchange of phospholipids.

IV. EXCHANGE OF PHOSPHOLIPIDS AND THE 105 000 × *g* SUPERNATANT

The 105 000 × *g* supernatant isolated from a perfused rat liver contains phospholipid (0.2 μg phospholipid-*P* per mg protein) [42]. It is presumed that this phospholipid is part of the lipid–protein complexes which are present in this supernatant [68]. It is not known whether these complexes are related to the lipoproteins secreted by the liver. The presence of serum lipoproteins in a total liver homogenate has been demonstrated using immunological techniques [69]. By similar techniques it has been recently shown that precursors of the serum very low density and low density lipoproteins are present in the Golgi body [70,71].

On incubation of the 105 000 × *g* supernatant with ^{32}P-labelled microsomes or mitochondria from rat liver, the phospholipids in the supernatant became rapidly labelled as a result of phospholipid exchange between the soluble lipid–protein complexes and the labelled subcellular particles [42,66]. Akiyama and Sakagami [66] showed that on subsequent incubation of this in vitro ^{32}P-labelled supernatant with unlabelled mitochondria, label was transferred to the mitochondria. They concluded that the stimulatory effect of the 105 000 × *g* supernatant on the exchange of phospholipids between mitochondria and microsomes was due to the presence of the lipid–protein complexes in the supernatant. However, after a pH 5.1 adjustment of the supernatant precipitated about 95% of the phospholipid and 40% of the protein, it was found that the resulting material denoted as pH 5.1 supernatant, was as active as the original one in promoting exchange [42]. It was concluded, therefore, that apparently a factor distinct from the bulk of the lipid-containing proteins was operative in the observed exchange between membranes.

The effect of the pH 5.1 supernatant on the exchange of phospholipids between mitochondria and microsomes is shown in Fig. 1. On incubation of ^{32}P-labelled mitochondria with unlabelled microsomes, the decrease of specific activity of the mitochondrial phospholipids was almost proportional to the amount of pH 5.1 supernatant protein added. A concomitant increase of label in the microsomal phospholipids was seen. The mitochondria used in this experiment were isolated from rat liver 16 h after injection of [^{32}P]phosphate to assure a homogeneous labelling

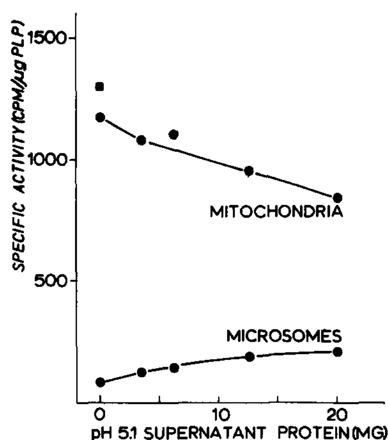

Fig. 1. The effect of various amounts of pH 5.1 supernatant protein from rat liver on the exchange of total phospholipid between ^{32}P-labelled mitochondria and unlabelled microsomes as reflected by the specific activities of mitochondrial and microsomal phospholipid. Mitochondria (52 μg phospholipid-*P*) and microsomes (96 μg phospholipid-*P*) were incubated for 40 min at 37 °C. ■, specific activity of mitochondrial phospholipid before incubation. For further experimental details, see ref. 42.

of the bulk of the mitochondrial phospholipids. Thus, the extent of mitochondrial phospholipid exchange can be calculated directly from the drop in specific activity. For example, a 40-min incubation at 37 °C in the presence of 20 mg pH 5.1 supernatant protein resulted in a 35% exchange of the mitochondrial phospholipids (see Fig. 1). McMurray and Dawson [55] observed that on incubation of mitochondria containing [^{14}C]phosphatidylcholine with unlabelled microsomes in the presence of supernatant, an isotopic equilibrium was reached within 2 h. By following the exchange of doubly labelled phosphatidylcholine (^{3}H label in the choline moiety, ^{14}C label in the fatty acid moiety at the 2-position) between mitochondria and microsomes, it was ascertained that the intact molecule was transferred in the course of the exchange process [72].

It was demonstrated by four groups of workers [73–76] that on incubation of mitochondria with ^{32}P-labelled microsomes, both the outer and inner mitochondrial membrane phospholipids became labelled. This would explain the very extensive exchange of mitochondrial phospholipids observed. However, the question still remained as to the manner in which the outer and inner mitochondrial membrane were involved in the exchange. Wojtczak et al. [76] contended that the inner membrane became labelled only by direct exchange with the microsomal phospholipids. This implied that the observed incorporation of label into the inner membrane was due to a detachment of the outer membrane. On the other hand, Blok et al. [74] provided strong evidence that the ^{32}P-labelled phospholipids enter or leave the inner membrane primarily via the outer membrane. It was demonstrated that phospholipids exchange inside the mitochondrion between outer and inner membrane. An exchange factor, however, comparable to that in the whole cell 105 000 × *g* supernatant

could not be detected in the mitochondrion. It was suggested that exchange of phospholipids inside the mitochondrion may be due to the outer and inner membrane being contiguous structures. Swelling of mitochondria in situ by perfusion of the rat liver with calcium acetate enhanced the transfer of labelled phospholipid from outer to inner mitochondrial membrane while the transfer between microsomes and total mitochondria was unaltered [77]. This observation reinforced the idea that the spatial relationship of the inner membrane relative to that of the outer membrane may effect the transfer of phospholipids inside the mitochondrion.

In addition to the exchange between mitochondria and microsomes, Kamath and Rubin [78] have shown that the pH 5.1 supernatant fraction from rat liver stimulated the exchange of phospholipids between individual populations of microsomal vesicles and between microsomes and plasma membranes. In general, then, it is very likely that all intracellular membrane structures are potential phospholipid donors and acceptors in the exchange process.

Various studies have shown that exchange of phospholipids is not limited to the liver membrane systems; in fact appears to be a rather widespread phenomenon. Miller and Dawson [79] observed a transfer of ^{32}P-labelled phospholipids from ^{32}P-labelled guinea pig brain microsomes to unlabelled brain mitochondria in the presence of a brain supernatant. In this study it was shown that the extent of exchange varied with the subcellular membrane particles present in the medium. Exchange of phospholipids was slow between microsomes and synaptosomes and could not be observed between microsomes and myelin. Furthermore, Jungalwala et al. [80] showed that the supernatant isolated from the thyroid gland of the pig stimulated the exchange of phospholipids between thyroidal microsomes and mitochondria. A similar exchange has been observed between these subcellular membrane fractions isolated from potato and cauliflower [81]. Again exchange was significantly stimulated by the cell supernatant of these plants. In this study it was emphasized that apparently the phospholipid–protein complexes present in the supernatant acted as intermediates in the exchange process.

With regard to the phospholipid-exchange activity of the pH 5.1 supernatant fraction from rat liver, there is no absolute requirement for rat liver membrane fractions. So far, this pH 5.1 supernatant has been found to stimulate the exchange of phospholipids between the following phospholipid-containing fractions:

(1) Rat liver mitochondria and the total lipoprotein fraction isolated from rat plasma [42]. (2) Rat liver microsomes and total rat plasma [41]. In this system a net transfer of microsomal phosphatidylcholine and phosphatidylethanolamine to the plasma was superimposed on the pH 5.1 supernatant-dependent exchange. (3) Rat liver mitochondria and systems such as liposomes, chylomicrons and artificial fat emulsions [82]. (4) Rat liver microsomes and liposomes [83]. (5) Liposomes sensitized with Forssman antigen and nonsensitized liposomes [50]. (6) Very low density and high density lipoproteins [84].

From this enumeration it follows that for the pH 5.1 supernatant to be active a natural membrane is not a prerequisite. In fact, it is suggested that a wide variety of

phospholipid-containing systems can be used and that no stringent structural requirements have to be met in these systems. It was, therefore, of interest to note that the rat liver pH 5.1 supernatant did not stimulate the exchange of phospholipids between human erythrocytes and rat liver microsomes or liposomes (Wirtz, K. W. A., unpublished observations). Preliminary experiments, however, have indicated that erythrocyte phosphatidylcholine is available for exchange provided the erythrocytes have been treated with sphingomyelinase from *Staphylococcus aureus* in order to remove sphingomyelin (Zwaal, R. F. A. and Wirtz, K. W. A., unpublished results). Thus it appears that the character of the interface involved in the process can affect the exchange.

V. SOME PROPERTIES OF THE pH 5.1 SUPERNATANT FRACTION

Phospholipid-exchange activity has been demonstrated in the cell supernatant of a wide variety of tissues such as rat [55,56,66], guinea pig [41] and beef liver [83]; rat [85], guinea pig [79] and beef brain [86]; rat [85] and beef heart [87]; rat kidney [85]; and pig thyroid [80]. Exchange activity was not detected in plasma of rat [41] and squirrel monkey [84]. From this it appears that the exchange activity is located intracellularly. The fact that the exchange activity is present in such a variety of tissues, underlines its role in the phospholipid economy of the cell.

By studying the exchange of the various classes of phospholipid it was observed that in the presence of a rat liver supernatant the exchange of phosphatidylcholine was faster than that of phosphatidylethanolamine between rat liver mitochondria and microsomes [55,65,66]. With these membrane fractions the exchange of phosphatidylinositol equalled that of phosphatidylcholine whereas a specific mitochondrial phospholipid, such as cardiolipin, did not exchange at all. In Fig. 2 the effects of rat liver and rat brain pH 5.1 supernatant fractions are compared with respect to the

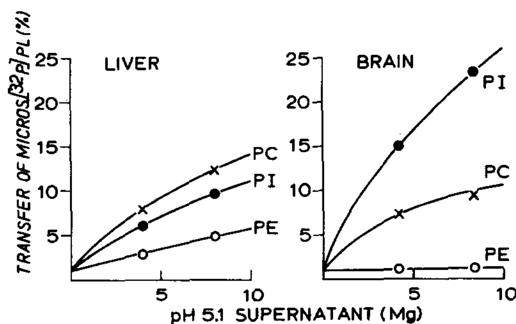

Fig. 2. The effect of various amounts of pH 5.1 supernatant protein from rat liver and brain on the exchange of individual phospholipids between ^{32}P-labelled microsomes and unlabelled mitochondria. Exchange was expressed as the percent of each microsomal [^{32}P]phospholipid pool transferred to the mitochondria. ×—×, phosphatidylcholine; ●—●, phosphatidylinositol; ○—○, phosphatidylethanolamine. For experimental details, see similar experiments described in ref. 86.

exchange of the various classes of phospholipid. Exchange of phospholipid is expressed as the percentage of each microsomal [^{32}P]phospholipid pool transferred to the mitochondron. It can be seen that for the brain supernatant the exchange of phosphatidylinositol relative to that of phosphatidylcholine is about three times faster than for the rat liver supernatant. Exchange of phosphatidylethanolamine was slow with liver supernatant, and not observed with brain supernantant. From this it may be concluded that it is the supernatant which determines to a large extent the relative rates at which each class of phospholipid is transferred. This has been confirmed in studies with pH 5.1 supernatant fractions of rat kidney and heart [85].

Whether the donor and acceptor membranes could affect these relative rates, is not well established. Miller and Dawson [79] observed that the soluble fraction of rat brain stimulated the exchange of each class of phospholipid to the same extent between rat brain mitochondria and microsomes. This is in contrast to what is observed if this brain supernatant is incubated with rat liver mitochondria and microsomes (see Fig. 2). This discrepancy might be due to the fact that donor and acceptor membranes were different in these two instances.

Since each class of phospholipid consists of a series of molecular species it was conceivable that the rat liver pH 5.1 supernatant stimulated the exchange of each molecular species to a different extent. This was also of interest in view of the hypothesis of De Pury and Colling [88] that the arachidonoyl-containing species of phosphatidylcholine was more firmly bound to the membrane than the other species. However, with regard to the molecular species of phosphatidylcholine it was shown that all the molecular species exchanged equally well between rat liver mitochondria and microsomes [89]. Taniguchi et al. [90] showed by isotope studies with rat liver slices that the rate at which the molecular species of phosphatidylcholine and phosphatidylethanolamine were transferred from the endoplasmic reticulum to the mitochondria was largely independent of the degree of unsaturation. Parkes and Thompson [91] came to a similar conclusion for the guinea pig liver. Upon administration of labelled glycerol and fatty acids to guinea pig they demonstrated that the distribution of label among the molecular species of mitochondrial phosphatidylcholine compared to that of microsomal phosphatidylcholine.

Phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine are present both in mitochondrial and microsomal membranes. In fact, the phospholipid composition of the outer mitochondrial membrane is very similar to that of the microsomal membrane [45]. It is not surprising, therefore, that the pH 5.1 supernatant promotes a real exchange of phospholipids between these membrane fractions. That is to say, as much phosphatidylinositol, for example, will be transferred from the microsomes to the mitochondria as in the opposite direction. The net result is that the phospholipid composition of the two membrane fractions remains unaltered during the exchange process. What will happen, however, if one of the membrane fractions lacks phosphatidylinositol? Will the pH 5.1 supernatant then redistribute the phosphatidylinositol between the two membrane fractions? That something like this might occur was indicated by an experiment in which ^{32}P-labelled microsomes

were incubated with liposomes consisting only of phosphatidylcholine, in the presence of beef liver pH 5.1 supernatant [83]. Under these conditions, both [^{32}P]phosphatidylinositol and [^{32}P]phosphatidylethanolamine were recovered in the liposomes. This demonstrates that the phospholipid-exchange activities present in the pH 5.1 supernatant have the potential to change the phospholipid composition of a lipid interface. It will be discussed in Section VIII, how phospholipid exchange might be a determinant of the overall phospholipid composition of a membrane.

Earlier experiments have demonstrated that the exchange activity of the rat liver supernatant was very likely a protein [42]. Activity was nondialyzable, precipitable with $(NH_4)_2SO_4$, sensitive to temperature and digestible by trypsin. It was recently shown by Illingworth and Portman [84] that sulfhydryl-blocking agents such as *N*-ethylmaleimide reduced the stimulatory effect of the rat liver pH 5.1 supernatant. It was suggested that one or more SH groups in the stimulatory protein were essential for activity. Furthermore, the experiments with rat liver and brain supernatant fractions implied that each supernatant might contain one phospholipid-exchange protein which differed from tissue to tissue in its ability to stimulate the exchange of the individual phospholipids (see Fig. 2). On the other hand it was conceivable that the exchange of each class of phospholipid was stimulated by a specific exchange protein. In this case it may be assumed that these specific proteins were present and/or active in the various supernatant fractions to different extents. To resolve this dilemma, isolation and characterization of the phospholipid-exchange proteins have begun.

VI. PHOSPHOLIPID-EXCHANGE PROTEINS

Suitable sources of phospholipid-exchange proteins have been the pH 5.1 supernatant fractions isolated from beef heart [87,92] and beef liver [72,83,93].

From beef heart a protein fraction with phospholipid-exchange activity was purified 82-fold using ammonium sulphate precipitation, hydroxylapatite adsorption–desorption and Sephadex G-100 fractionation [87]. On incubation of this protein fraction with ^{32}P-labelled mitochondria and unlabelled microsomes from rat liver, [^{32}P]phosphatidylcholine and [^{32}P]phosphatidylinositol were found to be transferred to the microsomes. In contrast to the pH 5.1 supernatant, the purified protein fraction lacked the ability to transfer [^{32}P]phosphatidylethanolamine, suggesting the removal of a phosphatidylethanolamine-exchange activity during purification.

Further attempts by Ehnholm and Zilversmit [92] to isolate a phospholipid-exchange protein from beef heart resulted in two active fractions of a high degree of purity. This was achieved by using ammonium sulphate precipitation, Sephadex G-75 fractionation and as a last step isoelectric focusing in a pH gradient from 4 to 6. One protein fraction was purified 179-fold; its major protein component had an isoelectric point of 5.5 and a molecular weight of 25 900. The other protein fraction was purified 295-fold, had an isoelectric point of 4.7, and the molecular weight of its major protein component was 21 000. Specificity of the protein fractions with respect

to phospholipid exchange was determined with antigen-sensitized and non-sensitized liposomes both of which contained equimolar amounts of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. It was found that both fractions catalysed the transfer of phosphatidylcholine and, to a much lesser extent, sphingomyelin between the liposomes. Transfer of phosphatidylethanolamine was not observed. It was not determined whether these protein fractions catalysed the transfer of phosphatidylinositol. A comparison, therefore, with the previously isolated protein fraction from beef heart, which catalysed the transfer of both phosphatidylcholine and phosphatidylinositol, was not possible (see above). It was of interest to note that the relative extents of phosphatidylcholine and sphingomyelin transfer between the liposomes was similar for the purified fractions as well as for the crude pH 5.1 supernatant from beef heart. This suggested that the exchange activity with regard to these two phospholipids resided in one protein in each of the isolated protein fractions.

Beef liver has been used as another source of phospholipid-exchange protein. Recently Kamp et al. [93] purified an exchange protein 2680-fold by using pH fractionation, ammonium sulphate precipitation, DEAE- and carboxymethylcellulose adsorption–desorption and Sephadex G-50 fractionation. The protein was homogeneous by the following criteria: dodecylsulphate–polyacrylamide gel electrophoresis, immunoelectrophoresis and isoelectric focusing. On incubation of the protein with ^{32}P-labelled microsomes and unlabelled mitochondria from rat liver, [^{32}P]phosphatidylcholine only was transferred to the mitochondria. With a slightly less purified protein this specificity with respect to phosphatidylcholine was also demonstrated in the phospholipid transfer between ^{32}P-labelled microsomes and liposomes consisting of egg yolk phosphatidylcholine and between ^{32}P-labelled mitochondria and liposomes consisting of egg yolk phosphatidylcholine (70 mole %) and rat liver phosphatidylethanolamine (30 mole %). Because of its specific action this protein was called phosphatidylcholine-exchange protein.

The phosphatidylcholine-exchange protein was isolated from the pH 5.1 supernatant of beef liver. This supernatant, however, as well as catalysing the transfer of phosphatidylcholine, also catalysed the transfer of phosphatidylinositol and phosphatidylethanolamine between rat liver mitochondria and microsomes (see Table II). It was of interest, therefore, to demonstrate that the transfer of the latter two phospholipids occurred independently of the phosphatidylcholine-exchange protein present in this pH 5.1 supernatant. In order to provide this evidence, a rabbit antiserum against the phosphatidylcholine-exchange protein was prepared. The γ-globulin fraction of this serum inhibited the activity of the isolated exchange protein completely. On incubation of the beef liver pH 5.1 supernatant with the antiserum γ-globulin fraction the phosphatidylcholine-exchange activity was inhibited at most by 62% while the phosphatidylinositol- and phosphatidylethanolamine-exchange activities were virtually unaffected (Table II). From these results it may be concluded that, in addition to the phosphatidylcholine-exchange protein, other activities responsible for the exchange of phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine are present in beef liver.

TABLE II

PHOSPHOLIPID EXCHANGE ACTIVITIES OF BEEF LIVER AND BRAIN pH 5.1 SUPERNATANT FRACTIONS BEFORE AND AFTER TREATMENT WITH THE ANTISERUM AGAINST PHOSPHATIDYLCHOLINE-EXCHANGE PROTEIN

Transfer of [^{32}P]phosphatidylcholine, [^{32}P]phosphatidylinositol and [^{32}P]phosphatidylethanolamine from ^{32}P-labelled microsomes to unlabelled mitochondria was expressed as the percentage microsomal [^{32}P]phospholipid pool transferred. For further experimental details, see ref. 86.

Supernatant fraction	Antibody	Transfer of [^{32}P]phospholipid per mg pH 5.1 supernatant protein (%)* Phosphatidylcholine	Phosphatidylinositol	Phosphatidylethanolamine
Beef liver	–	2.03	1.74	0.23
	+	0.77 (38%)	1.64 (94%)	0.23 (100%)
Beef brain	–	1.26	3.90	–
	+	0.97 (77%)	4.11 (105%)	–

* Numbers between parentheses give percent activity relative to control value.

Treatment of beef brain pH 5.1 supernatant with the above antiserum γ-globulin fraction caused inhibition of the phosphatidylcholine-exchange activity by 23% while the phosphatidylinositol-exchange activity remained unaltered (Table II). As was seen with rat brain supernatant (see Fig. 2), beef brain supernatant did not catalyse the transfer of phosphatidylethanolamine. Recently Helmkamp et al. [94] have isolated two proteins from beef brain both of which catalyse the transfer of phosphatidylinositol and, to a much lesser extent, phosphatidylcholine. One of these proteins had a molecular weight of about 29 000 and an isoelectric point of 5.2 while the other protein electrofocused at pH 5.5 and gave a molecular weight of approximately 30 000.

In summary, it can be concluded that a series of phospholipid-exchange proteins exist which have different activities with respect to the transfer of the individual classes of phospholipid. The phosphatidylcholine-exchange protein of beef liver has demonstrated a specificity for phosphatidylcholine under the incubation conditions employed so far, whereas the other four exchange proteins discussed above appear to be less specific. That is, the two proteins from beef heart catalyse the transfer of phosphatidylcholine and, to some extent, sphingomyelin while the two proteins from beef brain catalyse the transfer of phosphatidylinositol and, to a lesser extent, phosphatidylcholine. It is currently under investigation to what extent the specificity of an exchange protein can be modified. Integral to this study is how the activity and the specificity of the exchange proteins are dictated by the phospholipid composition of the membranes involved.

VII. PHOSPHATIDYLCHOLINE-EXCHANGE PROTEIN

A. Properties [93,95]

Purification of the phosphatidylcholine-exchange protein from beef liver was

complicated by the fact the protein had a tendency to aggregate, particularly at the later stages of purification. Aggregates in the form of filaments were visible at concentrations as low as 0.1 mg protein per ml. Lyophilization of the protein was impractical since the material would not redissolve. The protein was routinely stored in 50% glycerol at —20 °C under which conditions the protein retained its full activity for months. Incubation at 70 °C caused complete inactivation while incubation at 50 °C was without effect. The protein was remarkably resistant towards trypsin treatment; more than 60% of the initial activity remained after 5 h incubation at 37 °C. The exchange protein had no distinct pH optimum, transfer being identical between pH 3.5 and 8.5 (Wirtz, K.W.A. and Demel, R. A., unpublished observations).

It was calculated from the elution behaviour on Sephadex G-75 that the exchange protein had a molecular weight of 22 000. Electrophoresis on a dodecylsulphate–polyacrylamide gel in the presence of β-mercaptoethanol yielded a single band with an estimated molecular weight of 23 000. A molecular weight of 21 300 was computed from the amino acid composition. The protein had an isoelectric point of 5.8. From this it appears that the phospholipid-exchange proteins isolated from beef liver, heart and brain, all have molecular weights in the range of 20 000–30 000 and isoelectric points between 4.7 and 5.8.

The amino acid composition is given in Table III. It is seen that the exchange protein consists of 190 amino acid residues of which 38% belong to the charged amino acids (lysine, histidine, arginine, glutamic acid, aspartic acid) and 38% to the nonpolar side-chain residues (tryptophan, isoleucine, leucine, valine, tyrosine, phenylalanine, proline). The 2 moles half-cystine were part of a disulphide bridge.

TABLE III

AMINO ACID COMPOSITION OF PHOSPHATIDYLCHOLINE-EXCHANGE PROTEIN

For experimental details see ref. 93.

Amino acid	Moles per mole protein
Asp	16
Thr	5
Ser	11
Glu	29
Pro	10
Gly	16
Ala	14
Val	16
Cys (½)	2
Met	4
Ile	6
Leu	15
Tyr	9
Phe	8
Lys	16
His	3
Arg	8
Trp	2

The N-terminal amino acid was found to be glumatic acid by both the Edman procedure and dansylation.

The exchange protein has an average hydrophobicity of 1109 cal per amino acid residue. This is calculated from the amino acid composition according to the method of Bigelow [96]. Using the data of this investigator it can be concluded that the exchange protein has a rather high average hydrophobicity relative to its molecular weight of 22 000. This suggests an excess of nonpolar residues which may tend to lead to intermolecular hydrophobic bonding and account for the observed tendency to aggregate.

Lipid analyses indicated that the protein contained phosphatidylcholine. Extraction of lyophilized exchange protein gave 0.4–0.6 mole of phosphatidylcholine per mole of protein. Lyophilization, however, may have hindered a complete extraction since analyses on the lipid extracts of nonlyophilized protein gave 1 mole of phosphatidylcholine per mole of protein (Kamp, H. H., unpublished observations). This value for the phosphatidylcholine content has been confirmed in studies where phosphatidylcholine bound to the protein equilibrated with highly labelled [^{14}C]-phosphatidylcholine present in monolayers (see section "Mode of action"). The extraction of phosphatidylcholine with organic solvents indicated that hydrophobic interactions are involved in the binding of phosphatidylcholine to the protein.

In spite of the phospholipid bound to the protein, the protein was not highly surface active (Fig. 3). It was found that the protein collected at the air–water interface until a final pressure of 12.5 dynes/cm was attained. For comparison, proteins such as high-density lipoprotein apoprotein, mitochondrial structural protein and

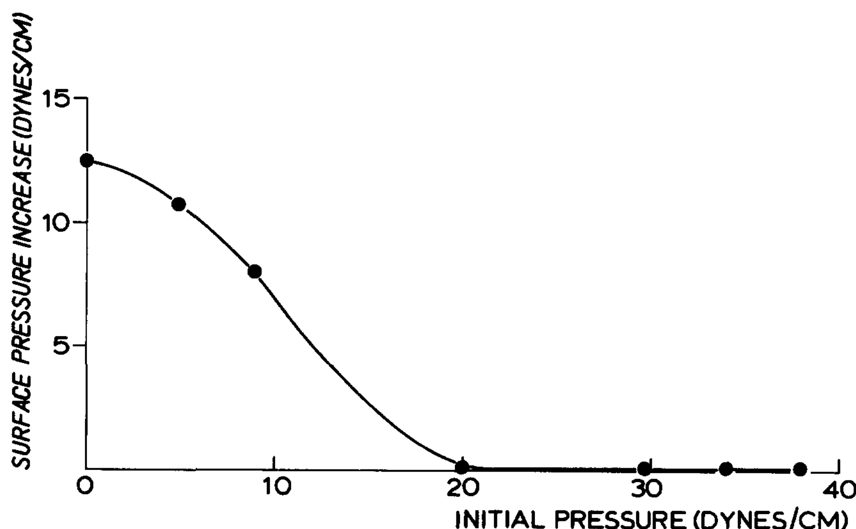

Fig. 3. Penetration of phosphatidylcholine (16 : 0/18 : 1) monolayers by phosphatidylcholine-exchange protein from beef liver at different initial surface pressure. The subphase consisted of 75 ml 0.01 M Tris–acetate (pH 7.4) and contained 48 μg (2.2 nmoles) exchange protein. For further experimental details, see ref. 95.

α-toxin have a saturation film pressure of 16–18 dynes/cm [97,98]. Furthermore, it follows from the increase of surface pressure depicted in Fig. 3 that the exchange protein could penetrate phosphatidylcholine monolayers provided the initial surface pressure of the lipid film was below 20 dynes/cm.

B. Mode of action

In order to investigate the mode of action of the phosphatidylcholine-exchange protein, Demel et al. [95] have made use of the phospholipid monolayer technique. Injection of exchange protein under monomolecular films of ^{14}C-labelled phosphatidylcholine diminished the surface radioactivity while the surface pressure remained constant. This suggested that phosphatidylcholine bound to the protein can exchange with phosphatidylcholine orientated at the air–water interface. It was calculated from the decrease of surface radioactivity, the amount of [^{14}C]phosphatidylcholine in the monolayer and the amount of exchange protein in the subphase that 1 mole of phosphatidylcholine per mole of protein was available for exchange. Chemical analyses of nonlyophilized protein gave the same phosphatidylcholine content. These data strongly suggested that the exchange protein acted as a carrier in the transfer of phosphatidylcholine between membrane structures. This hypothesis has proven to be correct with the aid of the same monolayer technique.

An experiment was designed to measure the transfer of [^{14}C]phosphatidylcholine between two separate monolayers, connected only through a common subphase (Fig. 4). Monolayer I contained [^{14}C]phosphatidylcholine (16 : 0/18 : 1) and Monolayer II the same, but unlabelled, component. Upon injection of exchange protein into the subphase, a transfer of [^{14}C]phosphatidylcholine from Monolayer I to II was seen. Since the initial surface pressure of both monolayers (30 dynes/cm) remained unaltered (not indicated in the figure) a concomitant transfer of unlabelled phosphatidylcholine occurred from Monolayer II to I.

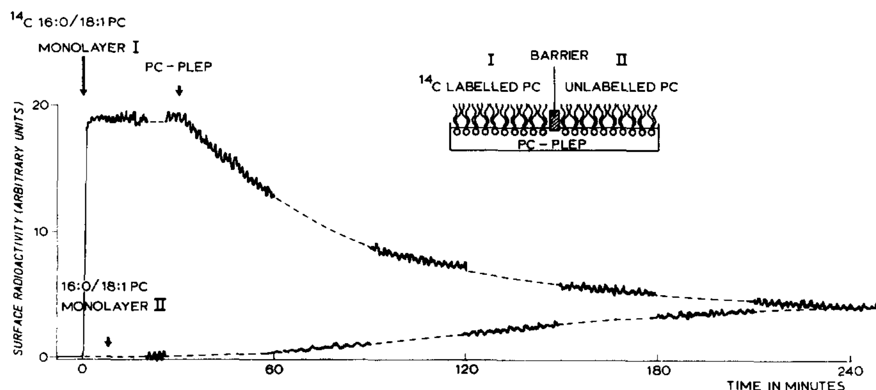

Fig. 4. Exchange of phosphatidylcholine between two separate monolayers, I and II. Monolayer I contained [^{14}C]phosphatidylcholine (16 : 0/18 ; 1) and Monolayer II the unlabelled component. The surface pressure of both monolayer was 30 dynes/cm. To induce exchange, 22.7 nmoles phosphalidylcholine-exchange protein (PC-PLEP) was injected in the subphase. For further experimental details, see ref. 95.

This experiment suggests the following sequence of events. The exchange protein collides with either monolayer, forming a transient collision complex. In this complex, phosphatidylcholine bound to the protein is replaced by phosphatidylcholine present in the monolayer. It is assumed that the interaction of the exchange protein with the monolayer is such that the protein penetrates up into the hydrophobic core of the monolayer. It is envisaged that the protein-bound phosphatidylcholine mixes freely with the monolayer phosphatidylcholine. During the process the ^{14}C label is distributed among Monolayers I and II and the phosphatidylcholine pool in the subphase contributed by the exchange protein. The specificity of the protein suggests that an electrostatic interaction between the protein and the polar head group of phosphatidylcholine plays a role in this process. Since transfer of label is only possible with a protein which moves freely through the subphase, the association constant of the collision complex should be low. Steady-state analyses have indicated that the association constant between the exchange protein and a single bilayer, phosphatidylcholine liposome is in the order of 0.1–5 $mmoles^{-1}$ (van de Besselaar, A. M. H. P. and Wirtz, K. W. A., unpublished). Due to the nature of the carried material i.e. long-chain phosphatidylcholine, the association constant between phosphatidylcholine and apophosphatidylcholine-exchange protein will be virtually infinite in an aqueous environment.

The exchange protein also catalyzed the transfer of phosphatidylcholine between a [^{14}C]phosphatidylcholine monolayer and phosphatidylcholine liposomes. The rate of exchange was independent of the film pressure as identical rates were observed at 20, 30 and 40 dynes/cm. This suggests that the formation of the collision complex as discussed above is not affected by the film pressure. The fatty acid composition of phosphatidylcholine in the liposomes, however, may play an important role. For instance, exchange of phosphatidylcholine was not observed with liposomes consisting of dipalmitoylphosphatidylcholine, in the subphase (Demel, R. A. and Wirtz, K. W. A., unpublished observation). It is currently under investigation as to what extent the crystalline–liquid crystalline state of a particular phosphatidylcholine species affects the exchange process. Liposomes consisting of sphingomyelin did not participate in the exchange with the [^{14}C]phosphatidylcholine monolayer confirming the specificity of the exchange protein (see Section VI). This specificity was also confirmed by the observation that the surface radioactivity of monolayers consisting of rat liver [^{32}P]phosphatidylinositol or [^{32}P]phosphatidylethanolamine did not diminish upon injection of phosphatidylcholine liposomes and exchange protein in the subphase.

VIII. POSSIBLE ROLE OF THE PHOSPHOLIPID-EXCHANGE PROTEINS

Thus far, phospholipid exchange activity has been demonstrated in a number of mammalian tissues (see Section V), in plants [81], and possibly in unicellular organisms such as *Tetrahymena pyriformis* [99]. These organisms have in common a great variety of intracellular membrane structures which can be distinguished both

morphologically and biochemically. For example, the various subcellular membrane structures present in rat liver have a distinct phospholipid composition [45,46]. At this stage one can only speculate as to what factors govern the phospholipid composition of a subcellular membrane. We suggest that the phospholipid-exchange proteins, as a group, may well be found to be one of these factors. An additional factor of obvious importance in controlling the phospholipid composition of a membrane would be the presence of enzymes involved in phospholipid metabolism, be it enzymes involved in de novo synthesis, base-exchange reactions, acylation–deacylation reactions, or degradation reactions. An extensive review of these enzymes has been recently published [100]. Furthermore, the protein constituents of a membrane may be of paramount importance in directing the type and quantity of phospholipid incorporated into a membrane at a given time [101,102].

In rat liver it has been demonstrated that the bulk of the subcellular membrane phospholipids, that is phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin is probably synthesized by enzymes present in the endoplasmic reticulum [100]. Isotope studies, however, showed that in vivo the newly synthesized phospholipids are very rapidly transferred to other subcellular membrane fractions such as the mitochondria [52,53]. Pulse-chase experiments [58] using isolated hepatic cells, radioautographic studies [57], and studies using drugs which interfered with the phospholipid metabolism of the endoplasmic reticulum, all strongly suggested that this rapid transfer of label was due to a continuous exchange of phospholipids between the endoplasmic reticulum and the mitochondria. Additional support for the microsomal and mitochondrial phospholipid pools being in a state of permanent exchange was provided by the observation that the half-life of each class of phospholipids was similar in microsomes, outer and inner mitochondrial membranes [55]. This has been confirmed for plasma membrane phospholipids which also had a half-life comparable to that of microsomal phospholipids [103]. In the latter study sphingomyelin was an exception in that turnover of this phospholipid was slower in plasma membranes than in microsomes. It is presumed that the phospholipid-exchange proteins are involved in the exchange of phospholipids observed in vivo.

What would the physiological significance of this continuous and fast exchange of phospholipids throughout the rat liver cell be? This question may relate to such basic cellular phenomena as turnover and membrane biogenesis i.e. growth. In this context, growth and turnover are thought to be related, in that an imbalance between rate of synthesis and rate of degradation will manifest itself in growth (or breakdown) [104,105]. For example, the proliferation of endoplasmic reticulum induced by phenobarbital might be explained in such terms [106]. In general, phospholipid turnover and membrane biogenesis will proceed continuously throughout the rat liver cell while phospholipid biosynthesis is predominantly localized in the endoplasmic reticulum. It is envisaged that the presence of the phospholipid-exchange proteins guarantees a permanent supply of phospholipid to those sites in the cell where a need exists. According to this hypothesis net transfers of phospholipids are

thought to be superimposed on the continuous exchange of phospholipids. Together, these functions suggest a fundamental role for phospholipid-exchange proteins in an intracellular homeostatic mechanism. In this concept, acylation–deacylation of phospholipids and base-exchange reactions involving phospholipids might provide a fine scale control of the membrane phospholipid composition in addition to other, as yet unknown physiological functions.

Mitochondrial biogenesis provides a good model to elucidate some of the points discussed so far [107]. Under unchanging conditions, the mitochondrial constituents of an adult rat liver will be in a steady state i.e. synthesis equals breakdown. Irrespective of whether or not a mitochondrion turns over as an entity, the mitochondrial constituents at large have been found to have a half-life of approximately 8–10 days [108–111]. In the situation, however, where partial hepatectomy brings on the growth of new liver tissue, the overall mitochondrial population will increase, i.e. biogenesis will surpass biodegradation. In both of the above physiological states a net transfer of phospholipids from the endoplasmic reticulum to the mitochondrial phospholipid pool will have to occur while exchange of phospholipids between the endoplasmic reticulum and mitochondria proceeds continuously.

It has been demonstrated that mitochondria have a limited capacity to synthesize some of their own proteins [112–116]. Evidence exists that it is in particular some of the hydrophobic proteins that are synthesized by the mitochondria [117–120]. Moreover, the mitochondria possess the enzymes to synthesize cardiolipin, a specific mitochondrial phospholipid [121,122]. Could it be, in fact, that the mitochondria are capable of synthesizing a "nucleus of crystallization" consisting of proteins and phospholipids, with which the mitochondrial proteins of cytoribosomal origin and the mitochondrial phospholipids of microsomal origin interact so that new mitochondria are formed? With respect to mitochondrial proteins of cytoribosomal origin, glutamate dehydrogenase, for example was found to associate preferentially with cardiolipin, suggesting that this phospholipid may play a role in the final location of this enzyme in the mitochondria [123,124]. The converse situation, namely the association of mitochondrial protein with phospholipids of microsomal origin, was demonstrated by Kagawa et al. [125]. They showed that a phospholipid-exchange protein isolated from beef heart transferred phosphatidylcholine from liposomes consisting of phosphatidylcholine to vesicles reconstituted from mitochondrial hydrophobic protein, cardiolipin and phosphatidylethanolamine. It is also of interest to note that phosphatidylserine decarboxylase activity is predominantly found in the rat liver mitochondria while the biosynthesis of phosphatidylserine takes place in the endoplasmic reticulum [126]. Dennis and Kennedy [126] suggested that perhaps phosphatidylserine may be transported to the mitochondrial site of decarboxylation by a phospholipid-exchange protein resulting in the formation of mitochondrial phosphatidylethanolamine.

Whether the phospholipid-exchange protein in the experiments of Kagawa et al. [125] actually parted with its presumably bound phosphatidylcholine without binding, for example, a phosphatidylethanolamine molecule in return, was not

ascertained. It is assumed that the exchange protein will deposit its bound phospholipid in the membrane only when the acceptor site effectively competes with the exchange protein for the phospholipid molecule [95]. It is thought that the hydrophobic properties of the acceptor site might be one of the parameters which determine this effectiveness. Experiments based on this hypothesis have been performed with high-density lipoprotein apoproteins isolated from human and rat serum. Although these lipoprotein apoproteins are known to bind phospholipids very avidly [97,127, 128], transfer of phosphatidylcholine from rat liver microsomes to these proteins in the presence of phosphatidylcholine-exchange protein was not observed (Moleman, P. and Wirtz, K. W. A., unpublished observation). This may indicate that the lipoprotein apoproteins lacked the proper conformation in an aqueous environment to interact effectively with the exchange protein. On the other hand it may be that an interface is a prerequisite for the phospholipid-exchange protein to exert its action (see section VII B).

Another important conclusion to be drawn from the experiment of Kagawa et al. [125] was that under appropriate conditions the exchange protein can redistribute a phospholipid, in this instance phosphatidylcholine, between a donor and an acceptor membrane. A similar observation has been made by Wirtz et al. [83] on incubation of a rat liver pH 5.1 supernatant with microsomes and liposomes consisting of egg yolk phosphatidylcholine. In this case, the transfer of phosphatidylinositol and phosphatidylethanolamine to the liposomes was observed. Preliminary experiments with the two phospholipid-exchange proteins isolated from beef brain indicated that these proteins can effect the net transfer of phosphatidylinositol from microsomes to liposomes deficient in this phospholipid (Harvey, M. S., Helmkamp, G. M. and Wirtz, K. W. A., unpublished). It is currently under investigation as to how the characteristics of the interface itself control the transfer of phospholipids as catalyzed by the exchange proteins to and from this interface. In the transfer of phosphatidylcholine from mitochondria to liposomes it was noted that the activity of the phosphatidylcholine-exchange protein was inhibited by increased negative surface charge of the liposomes [129]. This suggests a regulatory mechanism in that the phospholipid composition of the acceptor membrane affects the activity of the exchange protein. Since a series of phospholipid-exchange proteins appears to exist the question may be raised as to how the activity of each exchange protein relates to the final phospholipid composition of a suitable acceptor membrane. At this stage of our knowledge it would be premature to try to answer this question. However, investigation of such a relationship should be pursued as it may be pertinent to membrane biogenesis in eukaryotic cells.

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