Lecithin Exchange Between Rabbit Erythrocyte and Serum

SOME CHARACTERISTICS OF THE EXCHANGE OF LECITHIN BETWEEN RABBIT ERYTHROCYTES AND SERUM

by

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to my mother and the memory of my father

and

to my wife, Mary Ann

whose support and encouragement throughout

my studies were essential, and deeply appreciated

ABSTRACT

The exchange of lecithin between rabbit erythrocytes and serum has been investigated. The erythrocyte lecithin was labelled *in vivo* by administration of $Na_3{}^{32}PO_4$ which resulted in the acquisition of [^{32}P] lecithin by the cells primarily by exchange, and *in vitro* by esterification with [^{3}H] palmitate, [^{14}C] linoleate or [^{14}C linolenate. The rate of exchange *in vitro* of erythrocyte [^{32}P] phospholipids was independent of specific serum lipoprotein fractions, but was related to the serum phospholipid concentration. Analysis of the *in vitro* erythrocyte inward and outward exchange rates of [^{32}P] lecithin revealed that only 55% of the cellular lecithin pool takes part in the exchange. A similar conclusion can be reached by comparison of the fractional exchange rates of the acyl- and ^{32}P-labelled erythrocyte lecithin, or by comparison of the ratio of acyl to ^{32}P radioactivity of the erythrocyte lecithin with the ratio found in the serum after exchange *in vitro*. These data indicated that the proportion of the acyl-labelled erythrocyte lecithin pool (± S.E.M.) capable of exchange was 53 ± 5%, 64 ± 12% and 95 ± 6%, when esterified with [^{14}C] linoleate, [^{3}H] palmitate and [^{14}C] linolenate, respectively. Intravenous injection of labelled erythrocytes showed that *in vivo* [^{14}C] linoleyl lecithin was somewhat less exchangeable than [^{3}H] palmityl lecithin, which exchanged at about the same rate as [^{32}P] lecithin. The differences in the proportion of the acyl-labelled erythrocyte lecithin pool undergoing exchange could not be attributed to the preferential exchange of a particular molecular species of lecithin, although the tetraenoic lecithin did exchange to a relatively greater degree. This suggests that cellular lecithin formed by esterification of linolenate is found only in the portion of the pool available for exchange, while that formed by esterification of linoleate and probably palmitate is distributed throughout the entire lecithin pool. The

fractional exchange rates of all labelled lecithins were greater in old erythrocytes than in young cells, but the relative differences in the fractional exchange rates of these labelled lecithins did not vary with red cell age. The fractional exchange rate of [^{32}P] lecithin of isolated erythrocyte membranes was three times that of the lecithin of intact cells. However, the difference in fractional exchange rates between [^{3}H] palmityl-lecithin or [^{14}C] linoleyl-lecithin and [^{32}P] lecithin is reversed in the membranes. These observations suggest that a portion of the total cellular lecithin pool becomes available for exchange only upon disruption of the erythrocyte, and may be located in a relatively inaccessible portion of the membrane such as the inner surface.

ABSTRACT

Nous avons étudié les échanges de lecithine entre le sérum et les érythrocytes chez le lapin. La lécithine érythrocytaire est marquée in vivo par administration de $Na_3{}^{32}PO_4$ ce qui entraîne l'entrée de [^{32}P] lécitnine dans les cellules, surtout par échange. Le marquage in vitro se fait par estérification avec le [^{3}H] palmitate, le [^{14}C] linoléate ou le [^{14}C] linolénate. Le taux d'échange in vitro des [^{32}P] phospholipides érythrocytaires est independant des fractions lipoprotéiques sériques spécifiques, mais il est relié á la concentration des phospholipides dans le sérum. L'analyse des quantités de [^{32}P] lécithine qui entrent et qui sortent des érythrocytes par échange in vitro révèle que seulement 55% du pool lécithinique cellulaire prend part à cet échange. La même conclusion est obtenue par comparaison des taux d'échange de l'acyl- et de la ^{32}P-lécithine érythrocytaire ou par comparaison du rapport entre acyle et ^{32}P dans la lécithine érythrocytaire avec ce même rapport dans le sérum après l'échange in vitro. Ces résultats montrent que la proportion du pool de lécithine érythrocytaire acyl-marquée (± erreur standard de la moyenne) capable d'échange est de 53 ± 5%, 64 ± 12% et 95 ± 6% selon que l'ésterification est faite avec le [^{14}C] linoléate, le [^{3}H] palmitate ou le [^{14}C] linolénate. L'injection, intraveineuse in vivo d'érythrocytes marqués que la [^{14}C] linoléyl-lécithine échange moins que la [^{3}H] palmityl-lécithine, laquelle échange à peu près à la même vitesse que la [^{32}P] lécithine. Les différences dans la proportion du pool de lécithine érythrocytaire acyl-marquée soumise à l'échange ne peuvent être attribuées à l'échange préférentiel d'une espèce moléculaire particulière de lécithine, bien que la lécithine tétraénoique soit échangée à un taux relativement plus grand. Ceci suggère que la lécithine cellulaire formée par estérification du linolénate ne se trouve que dans la portion du pool disponible pour échange, alors que la

lécithine formée par estérification du linoléate et probablement du palmitate est distribuée à travers le pool entier de lécithine. Les taux d'échange de toutes les lécithines marquées sont plus élevés dans les érythrocytes âgés que dans les jeunes globules, mais les différences des taux d'échange de ces lécithines marquées par rapport à la [^{32}P] lécithine ne varient pas avec l'âge du globule rouge. Le taux d'échange de la [^{32}P] lécithine des membranes érythrocytaires isolées est 3 fois plus élevé que celui de la lécithine des cellules intactes. Cependant, dans les membranes, la différence dans les taux d'échange entre la [^{3}H] palmityl-lécithine ou la [^{14}C] linoléyl-lécithine et le [^{32}P] lécithine est contraire. Ces observations suggèrent qu'une partie de la lécithine cellulaire totale devient disponible pour échange seulement après rupture des érythrocytes et que cette lécithine serait localisée dans une portion relativement inaccessible de la membrane, telle la surface interne.

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ABBREVIATIONS

PC	phosphatidylcholine (lecithin)
PE	phosphatidylethanolamine
PS	phosphatidylserine
PI	phosphatidylinositol
PA	phosphatidic acid
S	sphingomyelin
LPC	lysolecithin
LPE	lysophosphatidylethanolamine
FA	fatty acid
GPC	glycerylphosphorylcholine
VLDL	very low density lipoprotein
LDL	low density lipoprotein
HDL	high density lipoprotein
μCi	microcurie
mCi	millicurie
BSA	bovine serum albumin
TLC	thin layer chromatography

1. INTRODUCTION

The mammalian erythrocyte is an unusual cell since it is anucleate (1). It has a definite species-dependent life span (2). When it is first released into the circulation from the bone marrow as the reticulocyte, the cell contains only RNA (1), mitochondria, and microsomes (3). At this stage the red cell is still capable of lipogenesis (4,5). However the intracellular organelles are lost within 36 hrs. (1), and in the absence of the organelles, lipogenesis by the red cell does not occur (4,5). The pathways by which the mature erythrocyte may renew its phospholipids are quite limited. Our laboratory has been interested in the mechanisms by which the phospholipid renewal may occur particularly with reference to in vivo red cell age. Previous studies of the Lands esterification pathway have been published (6,7). The present study examines the pathway of exchange of phospholipid between the erythrocyte and serum particularly in terms of the participation of phospholipids derived from the Lands esterification pathway in this process. This study has been presented (8) and has led to a recent publication (9).

1.1 The Red Blood Cell - General Description

1.1.1 The Reticulocyte

The reticulocyte is the last stage of development of the erythrocyte before maturity and normally is the only immature form present in the circulation. It is more adhesive than the mature erythrocyte and this property may account for its tendency to remain in the bone marrow under normal conditions (10). The circulating reticulocyte matures in 1-2 days depending on the species (11) and comprises only about 1-2% of the total circulating red cells (10).

Because the anucleate reticulocyte still contains RNA, which is present mostly as free ribosomes (12), it retains the ability to synthesize proteins. Although it can produce various proteins (12-15), including those

of the membrane (14,15), most of the protein synthesized by the reticulocyte is globin, which along with heme, is necessary for hemoglobin production (1). The reticulocyte still possesses mitochondria and microsomes and therefore the electron transport chain and the Krebs cycle are still functional (3). There is also evidence that the reticulocyte can synthesize lipid de novo (5,16-18). The reticulocyte is 20% larger in volume (19), contains more water, and at least 50% more lipid than the mature erythrocyte (20,21). In addition to the reduction in volume and loss of water and lipid upon maturation, "stress" or macroreticulocytes have been shown to lose up to 25% of their hemoglobin (20,22). However, in other studies in which normal erythrocytes were separated according to age, the youngest, reticulocyte-rich fraction contained no more hemoglobin than any of the other fractions, all of which contained the same amount of hemoglobin (23-29). As the reticulocyte matures, it expells its mitochondria by the mechanism of extrusion. Vacuoles develop in an irregular fashion and surround individual mitochondria. As the vacuoles grow larger they join to form larger vacuoles containing several mitochondria. The membrane of these vacuoles then fuse with the plasma membrane of the cell, resulting in the expulsion of the mitochondria (26).

1.1.2 The Erythrocyte

The mature erythrocyte is a biconcave cell about 8.5 μ in diameter, 1 μ thick at the centre and about 2-5 μ thick at the rim. It contains no RNA or subcellular organelles (27) and thus is not capable of respiration. The erythrocyte depends mainly on glycolysis for its energy requirements (1). In addition the erythrocyte no longer has the ability to synthesize lipid de novo (5,16-18). As the mature erythrocyte ages, its metabolic activities decrease with a general decrease in activity of

its enzymes. It continues to lose water, lipid (1) and becomes more osmotically fragile (28). Although there is a small amount of random destruction (29), most erythrocytes survive to the end of their life span. In the rabbit the erythrocyte circulates for about 65 days (30), after which time it is destroyed in the reticuloendothelial system (31).

1.2 Erythrocyte Lipid Composition

1.2.1 Total Lipids

Since the erythrocyte is devoid of any organelles (27), essentially all of the cellular lipid is found in the plasma membrane. The lipid constitutes about 40% of the dry weight of the erythrocyte membrane (32). Table I represents the average values for the lipid content of 14 different species of erythrocyte, including man, taken from an excellent recent review (33).

TABLE I

Average Lipid Content of the Mammalian Erythrocyte

Lipid	Lipid Content (mgm/ml packed cells)	Standard error of mean	Range (mgm/ml)
Total Lipid	4.98	±0.2	4.0 - 6.1
Cholesterol	1.32	±0.05	1.0 - 1.5
Phospholipid	2.90	±0.3	2.6 - 4.0

Despite some variation for the phospholipid values, the lipid content of erythrocytes is remarkably consistent and similar for all the species. In general, of the total lipid content, neutral lipid accounts for about 30% (34,35). At least 80-90% of this has been reported to be free cholesterol, the remainder being made up of cholesterol esters, free fatty acids, and mono-, di- and triglycerides (34,36,37). Nelson agreed with these findings, but showed in a single experiment that when sheep erythrocytes were washed ten times with phosphate buffer, only cholesterol

could be detected (38). Phospholipid generally constitutes about 60% of the total lipid of mammalian erythrocytes with the remaining 10% accounted for by glycolipids (39).

1.2.2 Phospholipids

Phospholipids, forming 50-60% of the erythrocyte lipid (39), are an extremely important and complex type of lipid. In addition to the major classes of phospholipids listed below in Table II, there are other known classes of phospholipids [eg. phosphatidyl-serine derivatives (40) and cardiolipin (35)] as well as many unknown phospholipids which are present in trace amounts (41). Table II outlines the distribution of the major erythrocyte phospholipids of several typical mammalian ruminant and non-ruminant species. For each species the values given are the means of at least four separate studies which were compiled by Nelson (33).

TABLE II

Distribution of Erythrocyte Phospholipids

Species	Percentage of Total Phospholipids						
	LPC	Sph.	PC	PS+PI	PE	PA	Other
Rat	4.8	16.2	47.8	10.3	24.2	0.2	---
Rabbit	0.2	20.9	37.0	10.6	30.8	1.6	---
Man	1.6	29.8	30.6	14.8	27.2	1.8	1.5
Cow	---	52.5	6.4	13.0	22.8	0.4	3.4
Sheep	0.4	52.3	3.3	12.8	28.9	0.8	3.2

It is obvious that lysolecithin, phosphatidic acid, and other components are present in minor quantities in both ruminant and non-ruminant species. The relative amounts of phosphatidylethanolamine, and phosphatidyl-inositol and -serine are similar among the various species. On the other hand the relative lecithin and sphingomyelin contents are highly variable. In general, the ruminant erythrocyte has a low lecithin content, whereas

in the non-ruminant cell the lecithin content is high. The sphingomyelin content varies inversely with the lecithin content and so that percentage of choline-containing phospholipids (lecithin and sphingomyelin) is rather constant, generally about 45-65% of the total phospholipid (33).

1.2.3 Fatty Acids

The fatty acid composition of the erythrocyte has been of great interest for many years and has been extensively studied. The historical work has been thoroughly reviewed (39). Nelson (33) has provided an excellent review of the more recent data and the reader is referred to it for detailed information.

The erythrocyte appears to have a fatty acid composition which generally is not remarkably different from that of other tissues (33). Although there is considerable variation from species to species, there seems to be a basic difference between ruminant and non-ruminant species. In contrast to the non-ruminant erythrocyte, notably that of the rat, which contains a large amount of arachidonic acid, the ruminant erythrocyte contains little arachidonic acid and a somewhat reduced amount of palmitic acid and a correspondingly greater quantity of mono-unsaturated fatty acid, particularly oleic acid.

The fatty acid composition of erythrocyte lecithin varies according to the species, but in general palmitic, stearic, oleic and linoleic acid accounts for 80-90% of all the lecithin fatty acid (39,42-44). Recently, through the use of argentation chromatography and gas-liquid chromotography it has become possible to analyse the individual molecular species of erythrocyte lecithin (43-45). Table III gives the mole per cent composition of these species in man, rat and rabbit. In general the major molecular species are similar for each mammal. Any molecular species of lecithin

TABLE III

Major Molecular Species of Erythrocyte Lecithin.

Fatty Acids Position 1	Position 2	Man (24)		Rat (26)	Rabbit (24)
16:0	16:0	9.5	4.2	20.2	10.3
18:0	16:0	3.7	1.3	4.3	3.0
16:0	16:1	1.8	-	-	1.0
16:0	18:1	14.0	27.2	14.5	19.5
18:0	16:1	1.8	-	-	1
18:0	18:1	4.4	2.3	0.8	-
16:0	18:2	20.4	19.5	12.4	24.0
18:0	18:2	11.3	8.5	5.6	17.8
18:1	18:2	9.3	1.4	0.7	
16:0	18:3	1.0	-	-	)
18:0	18:3	1.0	-	-	)2.8)
16:0	20:3	3.9	2.1	0.4	)
18:0	20:3	1.3	0.5	0.3	)4.1)
16:0	20:4	6.6	8.5	9.4	2.0
18:0	20:4	3.4	3.6	7.5	1.4
18:1	20:3	-	0.4	0.8	-
18:2	18:2	-	0.6	2.7	-
16:0	20:5	-	0.8	0.5	-
18:1	20:4	-	1.1	0.8	-
16:0	22:6	-	2.7	4.0	-
18:0	22:6	-	0.6	2.3	-
18:2	20:4	-	0.7	3.0	-

which does not have a fully saturated fatty acid in position 1 of the molecule is quantitatively minor. Unsaturated fatty acids are generally in position 2. The only major fully saturated molecular species is dipalmityl lecithin which is quantitatively quite significant in the rat erythrocyte, and distearyl lecithin is absent. Individual polyunsaturated molecular species of lecithin are present in minor quantities possibly because there would be a need to avoid peroxidation in a system which actively is involved in oxygen exchange (45).

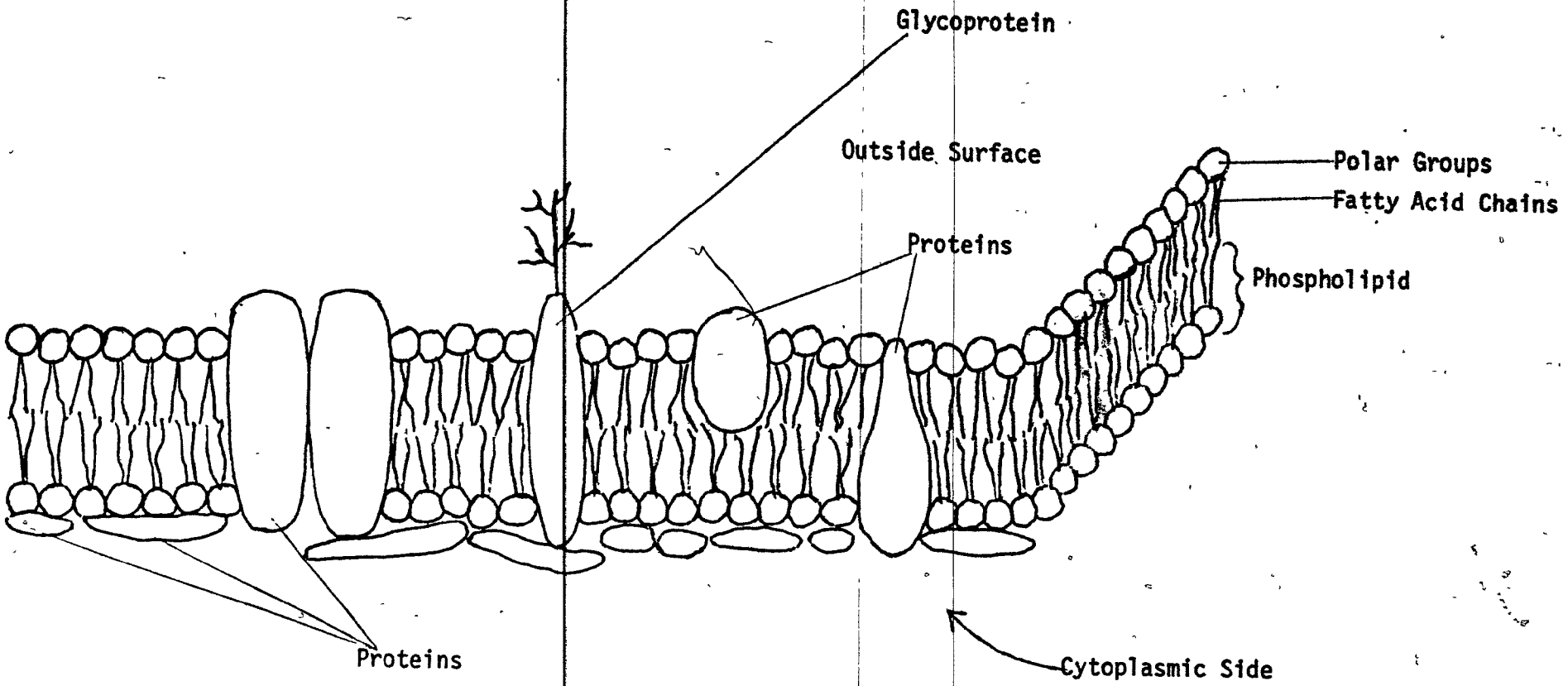
1.3 The Erythrocyte Membrane

1.3.1 Structure

In the time since Gorter and Grendel (46) and Danielli and Davson (47) first proposed the bilayer model for membrane structure many years ago, many models have been presented and reviewed (48,49). Recently Singer, after analysing these models (49), concluded that a fluid mosiac structure of alternating globular proteins and phospholipid bilayer is the only one consistent with thermodynamic restrictions and the known data (49,50). This new concept has since gained wide acceptance in general, and in terms of the erythrocyte membrane in particular (51-56). Because of this and the fact that a discussion of the many models of membrane structure is beyond the scope of this review, only the fluid mosiac model will be outlined.

In this model, the erythrocyte membrane is a lipid bilayer into which are imbedded molecules of globular proteins. These proteins may in one extreme be loosely associated with the surface of the bilayer, as is the case with spectrin, or in the other extreme penetrate the membrane completely from one side to the other as is the case with a 100,000 molecular weight protein and a sialoglycoprotein (53,54,56). A schematic diagram of the erythrocyte membrane is in Figure 1. This model requires that the

Figure 1. Fluid Mosaic Model* of Erythrocyte Membrane Structure (5)(9)



*Cholesterol Not Depicted

protein components be amphipathic, that is, be structurally assymetric with both polar and non-polar regions. The polar or hydrophilic regions, which would be relatively-rich in ionic amino acid residues and contain any protein polysaccharide residues present, would be preferentially located either on the external side or the cytoplasmic side of the membrane. The non-polar or hydrophobic portion of the protein, containing apolar amino acid residues could be associated with the lipid interior. The lipid bilayer is amphipathic as well, with the polar part of the phospholipid molecule externally oriented toward the aqueous environment, and the non-polar fatty acid moiety directed toward the centre. The concept of intrusion of proteins into the hydrophobic interior of the membrane is central for the model (50). In the case of the erythrocyte membrane, the sialoglycoprotein, for example, traverses the lipid bilayer with the polar amino-terminal part of the molecule and sialic acid residues on the outside of the membrane, and the carboxy-terminal portion, also containing polar amino acid residues on the cytoplasmic side. Spanning the membrane is a region high in α-helical content, containing about 30 amino acid residues. Most of them are hydrophobic and lie within the hydrophobic core of the membrane (55,56). A detailed description of the erythrocyte proteins, which is beyond the scope of this outline, has been given recently in an excellent review by Juliano (54). Up to 40% of human erythrocyte membrane protein is in the right-handed α-helical conformation (57) and Singer suggested that therefore the integral proteins of intact membranes are largely globular rather than spread out as a thin sheet (50). The peripheral proteins, which are only loosely connected with the membrane surface, may be bound to integral proteins as well as lipid. They are not thought of as being an important part of the membrane structure and are easily removed by merely washing with a chelating agent

or a buffer of high ionic strength (50).

Although the amino acid composition and the conformation of the proteins precisely determine their orientation with respect to and the degree of intercalation into the membrane surface, the proteins are quite mobile and may move about from one part of the membrane surface to another (50). Proteins account for no more than about 30% of the total membrane surface area (58). Thus, there is no extensive system or organization of proteins and hence no long-range order, although short-range local organization is likely. Thus, random distribution and arrangements or proteins in the membrane are the norm (50).

1.3.2 Lipid-Protein Interaction

Protein-lipid interactions appear to play an important role in membrane functions. Most membrane phospholipid is in the bilayer at a distance from any protein molecule, such that little if any interaction occurs. However, a small fraction of the lipid is adjacent to the protein and interacts with it. An interesting consequence of this is the fact that simultaneously in the membrane many phospholipid molecules interact very little if at all with protein, while other phospholipids interact very strongly with the protein (50). Both properties appear to be necessary and lead to various membrane properties. Extensive hydrolysis of the phospholipids of the intact erythrocyte into diglyceride with phospholipase C profoundly affects the physical state of the residual lipid but has no apparent effect on the average conformation of the membrane proteins (59). On the other hand, the membrane proteins of sheep erythrocytes, which contain a high proportion of sphingomyelin, preferentially bind sphingomyelin when presented with a mixture of lipids (60). In many membrane systems, membrane-bound enzymes require lipids, often specific phospholipids for activation (61).

Since the lipids and proteins of the membrane can be separated by organic solvents, their interactions must be weak and non-covalent, involving electrostatic or ionic interactions, hydrogen bonding, London-van der Waals forces and hydrophobic bonding (61a).

These interactions are probably important in the structural role of the different phospholipids in the membrane. Anionic phospholipids such as phosphatidylserine and phosphatidylinositol might be distributed differently from zwitterionic phospholipids, such as phosphatidylcholine, in areas near membrane proteins due to the influence of electrostatic interactions. These interactions are considered to occur through the side-by-side juxta position of anionic lipids and the cationic residues of protein and these interacting ionic groups would remain in contact with water. It is possible too that the difference in size of the zwitterionic group (compare lecithin with phosphatidylethanolamine) may also be the distinguishing feature between two otherwise very similar phospholipids (49). For proteins with apolar amino acid residues, as well as for lipids, exposure of apolar regions and groups to water is thermodynamically unfavourable. As a consequence, these groups tend to arrange themselves in such a way that they cluster together excluding water (thus forming hydrophobic bonds), therefore decreasing the energy state and increasing the entropy of the whole system (the membrane). Also, formation of these hydrophobic bonds results in increasing stabilization of peptide hydrogen bonds within these apolar areas, since water, which is a competitor of hydrogen bond formation, is excluded and the α-helix is the favoured conformation for hydrophobic polypeptides (61a). Thus, a lipid bilayer with an apolar core in intimate hydrophobic contact with the apolar amino acid residues of the membrane proteins is thermodynamically the most favoured structure. Zwaal and van Deenen in recombination experiments

of membrane lipids and proteins, concluded that both electrostatic and hydrophobic interactions were involved (62).

1.3.3 The Role of Lipid in the Membrane

In the fluid mosaic model the fluidity of the membrane is dependent on the lipid component. The fatty acid moiety of the phospholipids appear to be involved. The fatty acid chains may be in a relatively fluid state where they have a great deal of rotational and vibrational freedom or in a relatively closely packed ordered structure in which motion is restricted (51,54,55). At a particular temperature, the lipids pass through a phase change from the fluid to the liquid crystalline state or vice versa (54,55). The transition temperature of the membrane phospholipids is likely a function of the polar group, the fatty acid moiety, and the cholesterol content of the membrane (55). Generally, the shorter the chain length and the greater the degree of unsaturation, the lower transition temperature is. It has been suggested that as the saturated hydrocarbon chains get shorter, there is a decrease in London-van der Waals forces, allowing a greater mobility of the chains (63). This is basically true for phospholipids with unsaturated fatty acids as well (63), since such lipids have larger cross-sectional areas and therefore pack less effectively between cholesterol molecules and against protein molecules (51). Since phospholipids containing unsaturated fatty acids are more bulky than the saturated phospholipids, the bilayer containing these phospholipids will tend to be more permeable to non-electrolytes (51,55,63). The suggestion of variability in fluidity of some areas of the membrane compared to others might be advantageous, due to the fact that membrane functions requiring different degrees of fluidity might be operative simultaneously (55).

The concept of the asymmetrical distribution of phospholipids

across the erythrocyte membrane has been proposed recently (64), primarily based on the labelling of erythrocytes and ghosts with the non-penetrating reagents 4-aceto-4'thiocyano stilbene disulphonate (65) and formylmethionyl (sulphone)methylphosphate (64). Gordesky and Marinetti have since reached essentially the same conclusions using yet another non-penetrating probe, trinitrobenzenesulphonate (66). Zwaal et al. in 1973 reviewed (67) their own extensive studies as well as those of others on the differential hydrolysis of membrane phospholipids in intact erythrocyte and ghosts by various phospholipases. All of these investigations, and those which they have done since, which included the technique of freeze-etch electron microscopy (68), further substantiated the concept of membrane phospholipid assymetry.

The general view appears to be that the outer layer of the bilayer consists mostly of choline-containing phospholipids (53,55,68), although up to 20% of the cellular phosphatidylethanolamine may also be present (68). In erythrocytes with a low sphingomyelin content (e.g. guinea pig) the external layer will be mostly lecithin, whereas in erythrocytes with a low lecithin content (cow and sheep cells), the outer layer would consist mainly of sphingomyelin (68). In this context, it has been claimed that essentially only sphingomyelin can be detected by immunological means on the surface of sheep erythrocytes (69) and ox and sheep cells, in contrast to those of the guinea pig, are resistant to haemolysis by phospholipases A_2 and C (68). The inner layer consists mostly of phosphatidylethanolamine and phosphatidylserine although a small fraction of the choline phospholipids may also be present (53,64,68). Bretscher has suggested that protein may help make up the volume deficit of the inner half since more protein is associated with the inner surface of the erythrocyte membrane than the outer surface (53,64). He further speculated that because of the difference in fatty acid composition

of the various phospholipid classes, the fatty acid composition of the inner surface is more unsaturated than the external, resulting perhaps in a less ordered environment which might be better for accommodating proteins, compared to that of the outer layer (53).

For the proposed asymmetry to exist, it would have to be very stable. Any diffusion of phospholipids from one side of the bilayer to the other would have to be slow enough not to disturb the partition (64). Kornberg and McConnell have shown in synthetic bilayers that phospholipid molecules diffuse laterally across the surface very rapidly (70). The neighbour exchange rate was of about 10^{-6} seconds (71). However, migration of these phospholipids from one side of the bilayer to the other ("flip-flop") is very slow (at 30°C the half life is about 6 hrs.) (72). Bretscher (53,64) speculated that in the erythrocyte membrane the presence of cholesterol and proteins which penetrate both lipid layers will both decrease the rate of "flip-flop" to a point where the rate may be measured in weeks or months.

Cholesterol forms an integral part of the erythrocyte membrane and has a profound effect on its properties. Cholesterol and phospholipid interact closely in such a way that the long axes of the cholesterol and the hydrocarbon chains of the phospholipid are nearly perpendicular to the surface of the membrane (73-75). The interaction between the molecules is complex (73,75,76). It is thought that there is an ionic interaction between the polar group of the phospholipid and the 3-β-hydroxy group in the cholesterol molecule, but more dominant is the hydrophobic interaction of the phospholipid fatty acid chains with cholesterol (73,75).

Cholesterol apparently alters the fluidity of membrane fatty acid chains in a complex manner (77,78). As a result, at physiological temper-

atures the erythrocyte membrane is less fluid than the completely fluid or "melted" state but more fluid than the liquid crystalline or "ice-like" state (54,75). The latter states exist above and below the "transition" temperature respectively of pure phospholipids (54,55). The effect of cholesterol is achieved by a direct steric interaction of the cholesterol with the fatty acid moiety of the membrane phospholipids (75). The transition temperature of the saturated acids is decreased while that of the unsaturated fatty acids is increased (75,79,80).

van Deenen recently suggested that in a monolayer with cholesterol present, natural phospholipid species having one unsaturated fatty acid constituent present undergo a reduction in molecular size (63). This seems applicable to the erythrocyte membrane, since quantitatively most species of erythrocyte lecithin have the above structure (43,44) and the permeability of the erythrocyte membrane varies with the cholesterol content. The lipid bilayer is compressed and the permeability decreased when the guinea pig red cell is loaded with cholesterol (81,82). Conversely, cholesterol depleted erythrocytes become more permeable (83).

It has been suggested some time ago, that cholesterol is assymetrically distributed over the erythrocyte membrane surface, being concentrated around the periphery of the biconcave disc (84). Recently however, data has been presented which suggests that while it may have local organization, cholesterol overall is randomly distributed over the whole membrane surface (85). Obviously, this is a problem which needs further attention. In any case, the amount of cholesterol present in the erythrocyte membrane appears to affect the shape of the cell. The cholesterol content of the red cell can easily vary within a wide range with proportional changes in membrane surface area. Incubations of normal red cells with sera of varying

cholesterol concentration result in changes in erythrocyte cholesterol content, and there is a close correlation between these changes and osmotic fragility, which is a reflection of membrane surface area (86). Although the overall picture is still unclear, the increase in cholesterol content in certain diseases may be responsible for the formation of abnormal and sometimes bizarre changes in red cell shapes. In hepatocellular disease the cholesterol content is greatly increased, sometimes up to 75%, with no concomitant increase in phospholipid content (87). The erythrocyte becomes "spurred" or covered with spike-like projections (88,89). Obstructive jaundice is accompanied by the formation of so-called target cells. This "targetting" of the erythrocyte may be due to a 25-50% increase in cellular cholesterol accompanied by percentage increase in phospholipid which is about half of that of cholesterol (90,91).

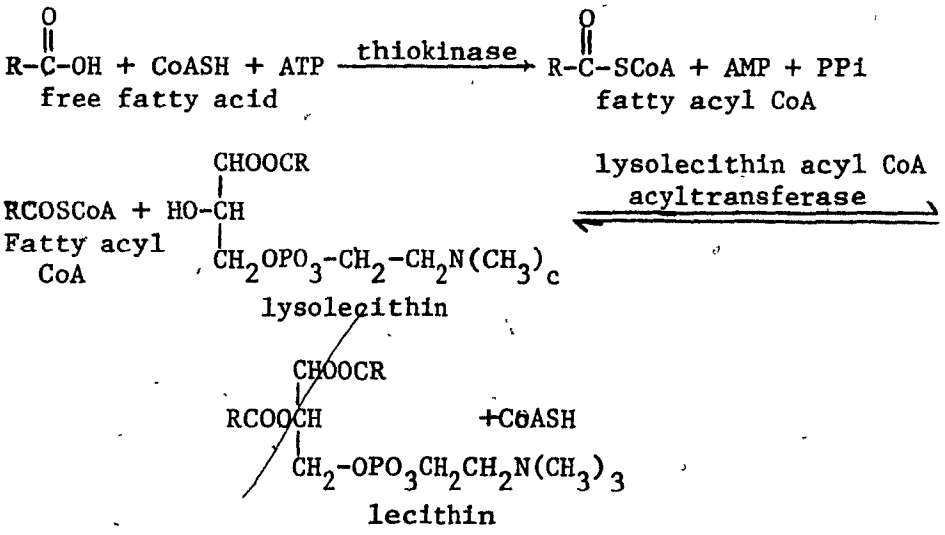
1.4 Erythrocyte Lipid Renewal and Metabolism

Upon maturation from the reticulocyte stage, the erythrocyte loses its capability for lipid biosynthesis de novo which includes that of fatty acid and other neutral lipids (7,17,92,93), phospholipids (17,93), and cholesterol (94). Fatty acids are not synthesized de novo from acetate due to the absence of acetyl-CoA carboxylase (4,16). There is no de novo pathway in mature erythrocytes for the synthesis of phospholipids due to loss of CDP choline:1,2-diglyceride phosphocholine transferase and CDP-diglyceride:inositol phosphatidyltransferase (5). Despite the loss of these important pathways the erythrocyte does have three major mechanisms of lipid renewal available to it: esterification of fatty acids with lysolecithin (7,95-97), exchange of cellular phospholipid with plasma phospholipid (98-100), and exchange of free cellular cholesterol with that of plasma (101-103).

1.4.1 Esterification of Fatty Acids

1.4.1.1 Mechanism

The pathway in which free fatty acid is esterified with lysolecithin was originally proposed by Lands in rat liver microsomes in 1960 (104) and first detected in erythrocytes by Miras et al one year later (105). The reaction is as follows:



The Lands Esterification Pathway

Miras et al showed that in addition of phospholipid, there was great incorporation of fatty acid into neutral lipids. However, Winterbourn and Batt (112a) have shown that leucocytes incorporate fatty acid much more rapidly than erythrocytes and that all of the radioactivity found in the neutral lipids could be accounted for on the basis of leucocyte contamination. Therefore, since Miras et al used whole blood, their results are questionable and one must also suspect that the erythrocytes of those workers who reported incorporation into neutral lipids, may have been contaminated with leucocytes (105-109).

It has been demonstrated that rat and rabbit erythrocytes which are high in lecithin, incorporate fatty acid to a greater extent than human

erythrocytes which have a lower lecithin content. Ovine and bovine erythrocytes, which contain very small amounts of lecithin, incorporate very little fatty acid (96,110). It has been suggested that these differences are more due to the relative permeabilities of the erythrocyte membrane to free fatty acid than to the relative abundance of the receptor for this precursor, since hemolysates of rabbit and ox incorporate fatty acid at the same rate (96).

The mechanism by which free fatty acid is taken up by the erythrocyte membrane and esterified has been extensively studied. Goodman (111) originally suggested that free fatty acids are transfered from serum albumin to the receptor binding sites on the surface of the cell, all of which have the same binding affinity, and in a manner which is independent of metabolic activity (111). Donabedian and Karmen (108) have also suggested that this occurs and that it constitutes the first step of the mechanism of fatty acid esterification. This step is rapid and reversible and the distribution of free fatty acid between albumin and the cells depends on the relative amount of each. Additionally, the distribution for each individual fatty acid is different and appears to be unrelated to the esterification rate for each acid. The rate of esterification is proportional to the fatty acid concentration in the medium. The second step involves the actual incorporation by esterification. Shohet et al (112) have studied the overall mechanism in detail. In agreement with the previous studies they suggest that the transfer of fatty acid from albumin to the superficial membrane pool "F1" is independent of cellular energy, and is extractable by defatted albumin. The second step, which involves transfer of free fatty acid from pool "F1" to "F2" apparently requires ATP. Fatty acid in pool "F2" is inaccessible to removal by albumin. The authors did not suggest how the cellular energy required for the transfer is spent, since the fatty acid in this pool is still

unesterified. However, they did state that the "F1-F2 step" may involve an opening up of the membrane allowing the third step, the actual esterification, which also has a requirement for ATP, to proceed at a maximal rate. Shastri and Rubinstein (6) also suggested that the incorporation step may be rate-limiting. Shohet et al calculated that the total energy cost of esterification to the cell is 5% of that derived from glycolysis (112).

When erythrocyte "ghost" preparations are incubated with free fatty acids, ATP and CoA must be added to the medium for esterification to proceed (96,110,113). Intact erythrocytes require no added cofactors (2,7) although one report claims otherwise (113). Such a case would require the transfer of the very large CoA molecule across the membrane, an unlikely situation.

The esterification enzyme, lysolecithin acylCoA acyltransferase has been demonstrated to exhibit specificity both with respect to the fatty acid precursor (96,97,108,110,114) and to the position on the lysolecithin molecule to which the fatty acid is esterified (95,96,113,114). In general for rat, man and rabbit, linoleic acid is esterified more rapidly than any other fatty acid (96,97,108,110,114), although in rat arachidonic acid is esterified equally fast (97). As in fatty acid composition (115), the rat appears to be the exception. Although linoleic acid is in each case esterified the most rapidly, the exact order of relative esterification rate for the other various fatty acids appears to vary considerably with the species (96,97,108). In general, however, the unsaturated acids are esterified more rapidly, and lauric (12:0) and myristic acid (14:0) are esterified very little, if at all (95,96,97,108). Bovine erythrocytes esterify linoleic and linolenic acid equally well, and oleic and arachidonic acid only somewhat less so (97). Thus, the transferase enzyme from

this source is not quite as specific, or there may be more than one enzyme.

Positionally, lysolecithin acylCoA acyltransferase esterifies the fatty acids at position 2 of the lysolecithin molecule, in most species (95,97,113). In the rabbit however, while 92% of the linoleic, and 84% of the oleic acid is esterified at position 2, only 9% of the palmitic acid is thus esterified. In other words, 91% of this fatty acid is esterified at position 1. Waku and Lands (97) have suggested that, for human erythrocytes at least, the cellular "acyltransferases could be the significant enzymatic factor controlling the fatty acid composition at the 2-position". These workers successfully predicted the fatty acid composition of this position from a knowledge of the esterification rates and plasma free fatty acid composition.

1.4.1.2 Diet and Other Extracellular Effects

Many studies have been carried out on the effects of various fat diets on erythrocyte lipids. These diets have included butterfat, which contains large amounts of short chain fatty acids, cocoanut oil (50% lauric acid), castor oil which contains 80% 12,OH-octadec-9-enoic acid, a non-mammalian fatty acid (116), corn oil (59% linoleic acid) (117), and other diets which are fat free. As early as 1959, it was found that a corn oil diet doubled the erythrocyte linoleic acid content over a matter of two years and it was assumed that this was due to incorporation during synthesis of new cells (118). However, it was soon shown that the 18-22 carbon unsaturated fatty acid content could be elevated with a similar diet in 10 days (119). In 1963 Farquhar and Ahrens (120) noted that equilibrium between dietary and erythrocyte linoleic acid is achieved in 4-6 weeks, a rate slower than that for plasma but higher than that of adipose tissue. No change in total lipid, phospholipid or cholesterol content was noted.

Mulder et al (96) found that when the diet of a rabbit maintained on cocoanut oil (lauric acid) was switched to corn oil (linoleic acid), the linoliec acid content in the red cell doubled within 10 days, mainly in lecithin and phosphatidylethanolamine.

Significantly, despite the cocoanut oil diet, the lauric acid content was never significantly increased although the plasma level was. Both Farquhar and Ahrens (120) and Mulder et al (96) noted that changes in oleic acid content were reciprocal with those of linoleic acid. It was also concluded that these changes occurred rapidly enough that it could not be solely due to maturation of the red cells in the bone marrow (96,120). A butterfat diet, which is rich in short chain fatty acids does not cause a change in the erythrocyte short chain fatty acid composition (116). In fact, fat free diets and diets deficient in linoleic acid in general result only in a very limited change in the erythrocyte fatty acid composition. The decrease in linoleic acid content is accompanied by a corresponding increase in the oleic acid content. Additionally, the concentration of arachidonic acid decreases (42,121,122), and this is due to the fact that, in the rat for example, arachidonic acid is synthesized from linoleic acid. This ability is reduced or missing in the sheep, and the arachidonic acid concentration does not vary (24). Also in essential fatty acid deficiency, eicosotrienoic acid (20:3) appears and it is normally absent from the erythrocyte (42,116,121-123). It has been suggested that these changes in erythrocyte fatty acid composition are restricted mostly to lecithin and phosphatidylethanolamine (42,122,124), but it has also been pointed out that the changes in erythrocyte fatty acid composition with diet is also species-dependent, particularly when comparing ruminant erythrocytes with non-ruminants (121). There is little doubt that in the

specific fatty acid changes the specificity of the lysophospholipid acyl CoA acyltransferase enzymes of the erythrocyte is heavily implicated (96, 121,122,124). This is further reinforced by Waku and Lands observations that the fatty acid composition can be predicted from a knowledge of the enzyme specificity and the levels of the various fatty acids in plasma (97).

As well as changes in the extracellular concentration of fatty acids there are other factors which can affect fatty acid esterification. Several groups of workers have reported that addition of lysolecithin to the medium in which either hemolysates (96,113,114) or intact erythrocytes (6) were incubated, stimulated the rate of fatty acid esterification. It was suggested that lysolecithin acted by making the membrane more permeable to fatty acid (6). Oliviera and Vaughan (95) however, could not show this effect with hemolysates and suggested that the endogenous level of lysolecithin was sufficient to saturate the system.

Several potential inhibitors of the esterification pathway have been studied. Cupric ion has been shown to be an effective inhibitor (95, 125), as has p-chloromercuribenzoate (126). The evidence concerning NaF is conflicting (110,126).

1.4.2 Exchange of Phospholipid

Exchange of phospholipid between erythrocytes and plasma was first described many years ago by Hahn and Hevesey (98). They suggested that the process was probably slow, that not all of the phospholipids of the erythrocyte were involved and speculated that perhaps the non-exchangeable fraction was located in the "deeper layers of the corpuscle membrane". 15 years ago several papers on phospholipid exchange were published, in which labelled acetate was used as a tracer in whole blood

(127-130). However, these same authors also showed that most of the acetate was incorporated by the leucocytes (131) and this is consistent with the current view that mature erythrocytes are incapable of incorporating acetate into lipids (4). Thus, their results must be interpreted with considerable caution.

Currently, it is considered that since erythrocytes are incapable of synthesizing lipids de novo (4,5) and since during exchange there is neither gain nor loss of the exchanging phospholipid from the cell (100, 132), phospholipid exchange between the erythrocyte and serum involves the intact molecule of the cell and its corresponding species in the serum (99,100,126,132,133). Except for the lyso derivatives used in the esterification pathway, it has been shown that a variety of possible phospholipid precursors are not incorporated (126,134). These include inorganic phosphate, CDP-choline, choline, ethanolamine, serine, inositol, glycerol, diacylglycerol, glycerylphosphate, phosphoryl choline, and glycerylphosphorylcholine. The various phospholipid classes exchange at different rates depending on the species of erythrocyte. In man and dog, Reed has shown that erythrocyte lecithin and sphingomyelin exchange with their corresponding species in plasma, but phosphatidylserine does not exchange, and phosphatidylethanolamine only slightly so. He suggested that the latter two phospholipids do not exchange well due to the lack of these classes in plasma (100). The findings were essentially confirmed by Shohet (126), although he showed that phosphatidylethanolamine is somewhat more exchangeable. Additionally, he found that lysolecithin, lysophosphatidylethanolamine, and phosphatidylinositol did not exchange at all. Mulder and van Deenen (134) reported that rabbit erythrocytes exchange mainly lecithin but also some lysolecithin with serum. When sheep erythro-

cytes were incubated with ^{32}P-labelled rabbit plasma there was only a very slight transfer of phospholipid, mainly as lysolecithin into the cells (134). The rat erythrocyte appears to be somewhat unusual in that lysolecithin is the main phospholipid participating in exchange and it exchanges extremely rapidly (99,133-136). Sphingomyelin and lecithin also exchange but at a much slower rate (99,134-136). It has very recently been implied that lecithin is the main phospholipid exchanged between serum and the erythrocyte of the pig (137).

With the exception of lysolecithin, which in the rat is exchanged almost instantaneously (133), phospholipid in general is exchanged quite slowly, about 1%/hr. (98,100,133), in a manner independent of metabolic energy (133).

It has long been suspected that exchangeable and non-exchangeable pools of erythrocyte phospholipid exist (98,130,134), but they have been quantitated only recently for two species (100). For example, Reed found that in man and dog, only about 60% of cellular lecithin is available for exchange. In human cells only 30% of the sphingomyelin is exchangeable, while dog erythrocyte sphingomyelin is 75% exchangeable (100).

Since the fraction of serum lipoproteins which has a density greater than 1.21 (including albumin) contains at least half of the total serum lysolecithin (138) it is not surprising that rat erythrocytes have been found to exchange lysolecithin mostly with this serum lipoprotein fraction (136,139). Other observations indicate that other cellular phospholipids, mainly lecithin and sphingomyelin preferentially exchange with those of high density lipoprotein (HDL, d = 1.063 - 1.21) (136,139,140). This would imply that there are two phospholipid pools in the serum. Reed has made an intensive study of the characteristics of lecithin and sphin-

gomyelin exchange between the human erythrocyte and serum both *in vitro* and *in vivo*. His data is consistent with a system in which the erythrocyte has two pools and the serum has only one, which is entirely exchangeable (100).

Reed (100) has suggested that one of the major factors affecting exchange of phospholipid between the erythrocyte and serum is the presence of the participating phospholipid in the serum. Of course, this is necessary for exchange of any phospholipid to be possible, but by the same token the phospholipid composition of the erythrocyte is likely to have a bearing on exchange as well (134). No mention has been made of the effect of varying the concentration of lipoprotein in the serum on the rate of phospholipid exchange. Within a given phospholipid class, the relative abundance of a particular molecular species in the cell or serum may have an effect. As well as these quantitative factors, certain qualitative aspects have to be considered. The nature of the binding of a phospholipid molecule to a membrane protein, i.e., electrostatic interactions vs. hydrophobic bonding is likely to have an effect on the availability of the phospholipid molecule for exchange (61,100). Perhaps even more important in this respect may be the physical position of the phospholipid molecule in the membrane (43,100). In addition to an assymmetric distribution of certain phospholipids in the erythrocyte membrane (68) it is possible, although it has not been commented on in the literature, that certain molecular species of any given phospholipid may also be assymmetrically oriented in the erythrocyte membrane. Finally, the state of the membrane which may change as the erythrocyte ages may also have an effect on the exchange of phospholipid between the red cell and serum.

1.4.3 Other Pathways

In addition to the major pathways of lipid renewal available to the erythrocyte, there have been reported several minor pathways of varying degrees of importance.

Perhaps potentially the most important pathway of these is the Erbland-Marinetti pathway:

$$2 \text{ lysolecithin} \xrightarrow[\text{acyltransferase}]{\text{lysolecithin}} \text{lecithin} + \text{glycerylphosphorylcholine}$$

In this reaction, which was first described by Marinetti et al (141) for rat liver, two lysolecithin molecules condense to form one of lecithin and one of glycerylphosphorylcholine without any requirement for ATP or CoA (142-144). The most ideal conditions for this pathway to be operative significantly appear to be those in which the pH is acidic and the lysolecithin concentration is relatively high (145). Mulder and van Deenen (114) first reported the existense of this pathway in the erythrocyte, but it has been shown that under normal physiological conditions, it contributes little to the lipid renewal of the red cell, compared to the fatty acid esterification reaction (126,144).

Despite some reports to the contrary (146-148), it is generally agreed that inorganic phosphate is incorporated only slightly by the erythrocyte solely into phosphatidic acid (33,149-152), through the action of a diglyceride kinase (149,150), and into di- and triphosphatidylinositol (153). It is possible that in sheep erythrocytes other trace and as yet unidentified phospholipids may also contain phosphorus derived from inorganic phosphate (154). In the nucleated erythrocyte of fowl, inorganic phosphate is incorporated into most major phospholipids *in vitro* (155,156) and *in vivo* (157). *In vitro*, phosphatidic acid and phosphatidylglycerol had very high specific activities (155,156), but *in vivo* they were weakly labelled (157).

Phospholipase A activity has not been demonstrated in the untreated intact erythrocyte despite repeated attempts (95,110,113,134,158). Polonovski's group claimed recently that rat hemolysates treated with 1 M NaCl (159) and human erythrocytes and hemolysates treated with trypsin, deoxycholate, or 1 M NaCl (160), have phospholipase A activity. These authors have also suggested that human erythrocytes undergoing hemolysis after being subjected to a prolonged incubation in isotonic media with plasma or a NaCl solution, exhibit phospholipase A activity (161). The significance of this activity in relation to physiological conditions is uncertain.

Mulder and Van Deenen have demonstrated the presence of lysophospholipase in the rabbit erythrocytes and suggested that its activity may be significant in the event of failure of the esterification pathway (144).

In the rat liver, the pathway in which phosphatidylethanolamine is successively methylated at the amino group, through the mediation of S-adenosylmethionine to produce lecithin, has been demonstrated (162). However, in the erythrocyte, the methionine activating enzyme is absent and there is significant incorporation of ^{14}C-labelled methyl groups into lecithin, sphingomyelin, methylated phosphatidylethanolamines and to some extent lysolecithin, through the action of methyl transferase, but only when S-[Me-^{14}C] adenosyl methionine is added to the system (163).

Polonovski and Wald have recently suggested that the human erythrocyte contains a triglyceridase. In order to show activity it was necessary to use an hemolysate and to emulsify the substrate with Triton X-100. Serum lipoproteins inhibited the hydrolysis (164). Michell and Coleman reported a lipase which attacks 1,2-diglyceride formed after phospholipase C treatment of rat erythrocytes (165).

1.4.4 Cholesterol Exchange

Exchange of cholesterol, which takes place between the erythrocyte and serum lipoproteins, is a very rapid process, reaching equilibrium *in vivo* in about 8 hrs. (166). There is no energy requirement for the exchange (166), which involves free cholesterol but not esterified cholesterol (167). The erythrocyte can exchange its unesterified cholesterol with plasma, isolated lipoprotein preparations, and artificial dispersions of lecithin-cholesterol and -sterol (168-173). When erythrocytes are incubated with cholesterol-deficient serum or lipid dispersions, the cell membrane can become up to 60% depleted of cholesterol (171,172) with a concomitant increase in osmotic fragility and glycerol permeability (174). The original cholesterol content and fragility can be restored by incubation with plasma or lecithin-cholesterol dispersions (172,174).

Despite its rapid rate and lack of an energy requirement, the cholesterol exchange between the lipoproteins and the erythrocyte does not constitute a simple system. All of the low density lipoprotein (LDL) free cholesterol (171), but only about 60% of the high density lipoprotein (HDL) free cholesterol (170) and a variable amount of erythrocyte cholesterol (depending on the species) (83,167,171), is available for exchange. Apparently the non-exchangeable erythrocyte cholesterol is essential for cellular integrity (175). d'Hollander and Chevallier (103) have recently proposed a model in which the serum lipoproteins (HDL and LDL) exchange cholesterol with the erythrocyte and with each other as well. They also suggested that most of the cholesterol which is esterified in the serum by lecithin cholesterol acyl transferase comes directly from the erythrocyte, the remainder coming from HDL (103,176).

1.5 Serum Lipoproteins and Phospholipid Metabolism

1.5.1 Lipoproteins

All of the major serum lipids, which include free fatty acids, phospholipids, cholesterol, cholesteryl esters and triglycerides are transported complexed with protein. While the free fatty acids and lysolecithin are mainly bound to albumin, the other lipids are carried in the macromolecular serum lipoproteins. The protein and phospholipids serve as the solubilizing agent which keep the neutral lipids in solution as they are transported in the circulation (177), There are four major families of circulating lipoproteins. These are summarized in Table IV (178). The following abbreviations are used in this Table:

VLDL - very low density lipoprotein

LDL - low density lipoprotein

HDL - high density lipoprotein

S_f - Lipoprotein Flotation Rate in Svedburg units (10^{-3} cm/sec/dyne/gm) in a NaCl solution of density 1.063 gm/ml.

Chylomicrons originate in the gastrointestinal tract and serve to transport dietary triglycerides from the intestine through the thoracic duct into the bloodstream and eventually to the tissues. They consist mainly of triglycerides with small amounts of other lipids and protein, and likely are spherical in shape. The phospholipids along with the protein form the outer membrane of the spherical particle, the core of which contains mainly triglyceride, but also a small amount of cholesterol ester as well (179).

Very low density lipoproteins are a class of lipoprotein rich in triglycerides which are endogenous in origin (178). These spherical lipoproteins are heterogeneous in their physical properties, resembling chylomicrons in one extreme, and low density lipoprotein in the other. The

TABLE IV

Properties and Composition of Major Lipoprotein Families

	Chylomicrons	VLDL	LDL	HDL
Density (gm/ml)	< 0.95	0.95-1.006	1.019-1.063	1.063-1.21
S_f	> 400	20-400	0-12	-
Molecular Wt.	$10^9 - 10^{10}$	$5-10 \times 10^6$	2.5×10^6	200,000
Molecular Size (Å)	750 - 10,000	300 - 800	205 - 220	75 - 100
Turnover Rate (Half Life)	5-15 min	6-12 hr	2-4 days	3-5 days
Lipoprotein Constituents % Dry Weight				
Protein	1-2	10	25	45-55
Triglyceride	80-95	50-70	10	3
Free Cholesterol	1-3	10	8	15
Esterified Cholesterol	2-4	5	37	22
Phospholipids	3-6	15-20	22	30
Carbohydrate	?	<1	~1	<1

predominant lipid of this class is triglyceride and the density of individual VLDL particles is related to the relative amounts of triglyceride and protein present. The protein moiety is made up of a number of quantitatively major and minor components (180,181), which are also found in LDL and HDL (182). Structurally it is considered that VLDL is spherical in shape and has a neutral lipid core which is surrounded by a thin layer of protein and phospholipid (178).

Low density lipoprotein is particularly rich in cholesterol and its esters (Table IV). It is derived solely from the metabolism of VLDL in the serum (183). The LDL particle appears to be a spherically-averaged structure consisting of a lipid bilayer coated with a layer of protein and presumably phospholipid which are present on both the inner surface and the outer surface of the bilayer (184). This is in contrast to the total structure of VLDL and chylomicrons (178,179). In the LDL structure, the cholesterol moiety of the cholesterol esters is associated with the protein and some of the polargroups of the phospholipids are exposed to the plasma (184). LDL is physically heterogeneous, components ranging from the lighter triglyceride-rich VLDL-like particles in one extreme through to a heavier HDL-like component (185).

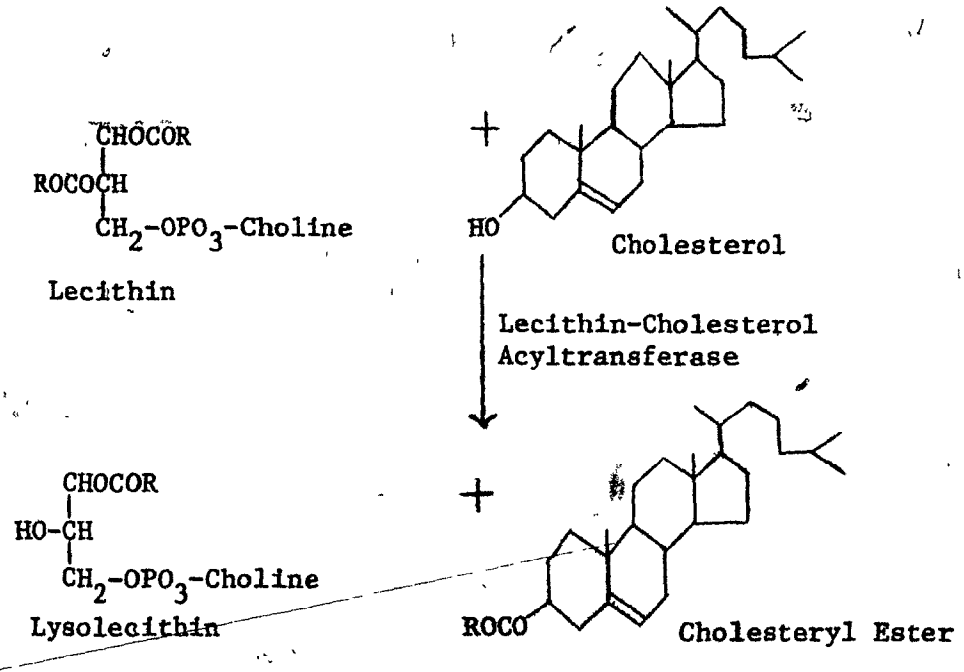
High density lipoprotein is present in the serum as two sub-classes HDL_2 (d.1.063-1.125) and HDL_3 (d.1.125-1.21). As a class HDL is higher in proteins and phospholipid than any other lipoprotein. The cholesterol content is also high although not as high as in LDL (Table IV). HDL_2 has less protein, triglyceride and free fatty acids, but more cholesterol than HDL_3 (186). It has been speculated that HDL may be a multi-unit structure (187). HDL is likely made up of physically similar or identical globular units probably linked together by lipids through non-polar bonding so that the

hydrophobic portions of the protein and phospholipids, as well as the neutral lipids are oriented away from the aqueous environment. In this thermodynamically stable structure the polar areas of the protein and the polar groups of the phospholipid are in contact with the water (187). A possible alternate structure for HDL has been suggested to be that of an organized system of micelles (187). HDL is secreted by the liver (188) and apart from lipid transport, its exact functional role is unknown (187). It is known that an HDL peptide is an activator of serum lipoprotein lipase (189) and that lecithin-cholesterol acyltransferase is associated with HDL (190).

1.5.2 Serum Phospholipid Metabolism

Phospholipid metabolism in the plasma appears to be quite limited. No esterification or condensation pathways analogous to those found in the erythrocyte have been observed. Lysophospholipase activity appears to be negligible (191,192). In post-heparin plasma of man a phospholipase which selectively hydrolyses phosphatidylethanolamine has been described (193,194) and in heparinized rat plasma a phospholipase hydrolyses lecithin significantly (195,196). These post-heparin phospholipases have little of no activity in normal plasma. By contrast, a similar enzyme has been found in normal human plasma which hydrolyses lecithin (197) and in normal rat plasma which hydrolyses phosphatidylethanolamine (192). The reason for these apparently contradictory observations is unclear. The contradiction may be real but it is also possible the heparin is having some direct effect on the phospholipase or is causing the release of another phospholipase, or both. In any case phospholipase substrate specificity appears to vary with the mammalian species. Indeed, it is possible that, in some species, there may be virtually no phospholipase activity in normal plasma.

One pathway of plasma phospholipid metabolism which has been documented for many different species (198,199-201) is the reaction first described by Glomset et al (202,203), the enzymatic transfer of fatty acid from lecithin to cholesterol.



This acyltransferase reaction is specific for lecithin only (204) and only the fatty acid in position 2 is transferred (203,205). Although there appears to be little specificity with regard to the species of sterol (206), the enzyme does show preference for certain species of fatty acids, depending on the species of animal (200,207-210). In rabbit plasma, the relative rate of transfer of fatty acids in descending order is linoleate > arachidonate > oleate > palmitate (200,208). The rate of linoleate transfer has been estimated at 6.8% over a 20 hr. incubation (208).

Under normal physiological conditions LCAT is well suited to catalyse transfer of fatty acid and from lecithin to cholesterol at a maximum rate. In rat (211) and human (212,213) serum at least, the optimum pH is between 7 and 8 and a pH below 6.0 inactivates the enzyme (198).

The reaction also proceeds more rapidly in isotonic saline than in either more dilute or concentrated salt solutions (213,214). Although LCAT readily becomes oxidized when partially purified, it appears to be quite stable under normal conditions in the plasma (198). On the other hand, if experimental conditions require the inactivation of the enzyme without changing normal plasma conditions, heating at 56-60°C for 30-60 min. is quite effective (215). LCAT is inactivated but the capacity of the plasma lipoproteins to act as substrates is unaltered (216).

1.5.3 Summary of Erythrocyte and Serum Phospholipid Metabolic and Renewal Pathways

The pathways of phospholipid renewal and metabolism are summarized in Figure 2.

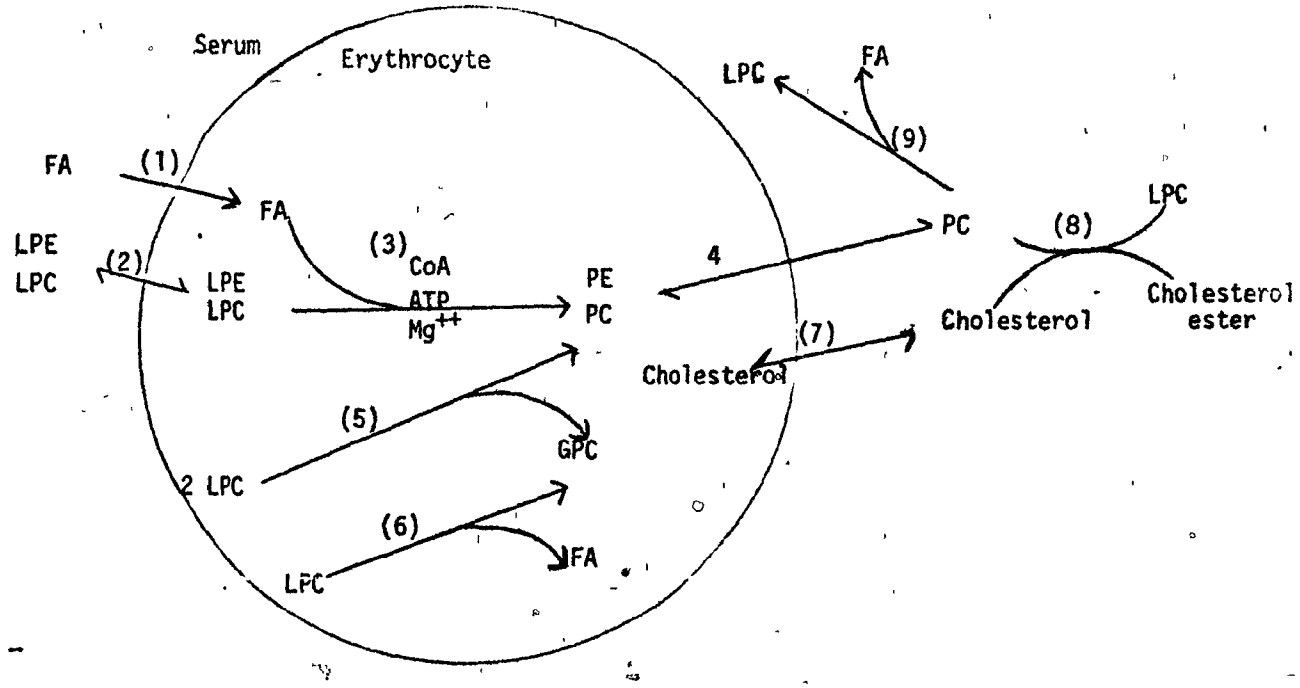
1.6 Membrane Alterations With Red Cell Aging

1.6.1 Total Lipid Content and Composition

The fact that the lipid content and composition change as the red cell matures and ages has been known for many years. Reticulocytes were first shown to contain more lipid than the mature erythrocyte in 1950 (217). Since then a number of studies have been made (7,20,21,218,219) and young reticulocytes have been reported to contain anywhere from 1.5 (21) to 4 (20) times as much phospholipid as mature cells. The contribution of lecithin loss to the overall loss of phospholipid upon red cell maturation seems to be in dispute. Raderecht (219) suggested that lecithin is preferentially lost, Shattil and Cooper (20) could find no change in cellular phospholipid composition, and Warrendorf and Rubinstein (7), reported that the lecithin content is greater in mature erythrocytes than reticulocytes. The loss of cholesterol is not as proportionately great as that of phospholipid (20,219). Reticulocytes have a lower cholesterol:phospholipid (C:P) ratio than mature

Figure 2

Schematic Diagram of Pathways of Phospholipid Renewal and Metabolism in the Erythrocyte and Serum.



(1) Incorporation of Fatty Acid (FA)
(2) Exchange of Lysolecithin (LPC) between Erythrocyte and Serum (rapid)
(3) Esterification of Fatty Acid (Lands Pathway) esterification of LPE much slower than LPC
(4) Exchange of Lecithin (PC) between Erythrocyte and Serum (not all PC derived from Lands Pathway may exchange)
(5) Erbland-Marinetti Pathway (not normally important)
(6) Lysophospholipase (not normally significant)
(7) Cholesterol Exchange (rapid)
(8) Lecithin-Cholesterol Acyl Transferase Pathway
(9) Serum Phospholipase A_2

erythrocytes (20,21) but the C:P ratio for their respective plasma membranes is the same (20). It has been suggested that much of the reticulocyte lipid is lost when the phospholipid-rich, cholesterol-poor organelles are lost from these cells (20,21), but Warrendorf and Rubinstein noted that these organelles contained little lipid when compared to the total lipid of the plasma membrane (7). Shattil and Cooper noted that the spleen also has a role in this lipid loss but the mechanism is not known (20).

After the erythrocyte matures it continues to lose lipid although the amount lost is relatively small (220-222). Loss of cholesterol is significant when expressed in terms of per single cell as well as per unit surface area (221,222). Total lipid and phospholipid loss appear to be significant only when expressed on a single cell basis rather than per unit surface area (221,222). Lipid loss from the mature erythrocyte as it ages appears to be on a proportional basis, such that the lipid composition of the cell does not change with age (222).

1.6.2 Red Cell Shape Alteration with Age

The biconcave shape of the erythrocyte is dependent on an excess of surface area relative to cell volume (223,224). This biconcavity lends to the cell an almost infinite ability to bend and deform (225) which the erythrocyte must do in passing through the narrow channels of the spleen (225,226). The erythrocyte membrane has only a very limited ability of stretch and the biconcave shape circumvents this problem (223). As the red cell ages, the lipid is lost (220-222), accompanied by a depletion of intracellular ATP (227,228), surface area is decreased (229), and the erythrocyte tends to become spherical (230). The erythrocyte in this shape has lost its deformability with the result that it is susceptible to entrapment and destruction in the microcirculation of the spleen (226). The adverse pH

conditions of the spleen may further aggravate the erythrocyte's lack of deformability (226) and the low levels of ATP likely reduces the deoxygenated senescent red cell's chances of survival of the effects of stagnation in the spleen in the same way (224,226).

Likely due to a loss of deformability of the membrane the osmotic fragility of the erythrocyte increases with age (231-232) and destruction of the senescent cell by osmotic hemolysis or by direct physical perforation likely occurs to some extent (229).

In an electron microscopic study of erythrocyte membranes, Danon and Perk (233) noted that the young membranes were granular, relatively thick, and generally had large concentric foldings, while membranes from old cells were "smooth and thin", less granular, and had mostly radial foldings. Later studies (232,234) showed the the young erythrocyte membrane contained a large number of long filamentous protrusions, which in old cell membranes were almost non-existant. These observations are consistent with the process of fragmentation, which Weed and Reed (229) have suggested as the mechanism for the symmetrical loss of lipid with increasing erythrocyte age. It had been previously suggested that membrane fragments may be lost repeatedly without haemolysis (225), with a resulting increasing sphering of the erythrocyte each time (229). While fragmentation itself may not be fatal to the cell, it may, along with decreasing ATP concentration (227,228) and possible loss of membrane-bound enzyme activity, predispose the cell to further fragmentation with the final deleterious consequence of inability to traverse splenic microcirculation (fragmentation hemolysis and/or phagocytosis) (229).

1.6.3 Fatty Acid Composition Changes

The fatty acid composition of the erythrocyte appears to change

with age. It has been noted the cells of patients with reticulocytosis are low in linoleic acid (235,236). An early study of age-related fatty acid composition changes indicated that in general the content of unsaturated fatty acids increase while that of the saturated fatty acids decrease with age (237). van Gastel et al (238) on the other hand, noted that while the linoleic acid content is slightly higher in old cells, the arachidonic content is decreased somewhat. In agreement with this, Phillips et al (239) recently found that four of the five fatty acids studied with chains of 18 carbons or less (stearic acid being the exception) increase in content with the age of the erythrocyte and all 19 fatty acids with 20 carbons or more show a decrease. The authors suggested that while the change in content of not every fatty acid longer than 20 carbon atoms is statistically significant, the direction of change of this group of fatty acids is very significant. Walker and Yurkowski (122) studied change in the erythrocyte fatty acid composition in erythrocytes from rats fed different diets. They found that significant differences occur with age on all diets and the pattern depends on the particular fatty acid and its concentration, and on the nature of the diet. They suggested that the fatty acid esterification pathway is involved and that the activity of the acyltransferase enzyme changes with the age of the erythrocyte.

1.6.4 Esterification of Fatty Acids

A number of other investigators have studied the esterification of fatty acids in relation to erythrocyte age. The reticylocyte incorporates fatty acid into lecithin at a rate which is four times that of the mature erythrocyte (7,240). Warrendorf and Rubinstein found that by far most of the fatty acid is incorporated into the plasma membrane but a very small amount is taken up into the mitochondria. Elevation of the ATP level by

50% or depletion of it to mature cell concentration has no effect. The authors concluded that although esterification of fatty acid by the reticulocyte is much more active than that by the mature erythrocyte, it follows the same pathway and is essentially restricted to the plasma membrane. Several laboratories (6,241,242), have shown that the oldest segment of the erythrocyte population esterify fatty acids at a reduced rate compared to the remainder. However, if the hemolysates of old cells are supplied with ATP (241) or the intact old cells are supplied with glucose (6), the difference in esterification rate is abolished. The reactivity of the acyltransferase system therefore appears to be dependent on the availability of endogenous ATP (6,241).

1.6.5 Other Lipid Pathways

Other pathways of lipid renewal and metabolism have remained largely unstudied in relation to the age of the red cell. Ferber et al (242) have reported that lysophospholipase activity is diminished by about half in old erythrocytes. Sloviter et al (94) have shown that cholesterol biosynthesis and exchange take place in the reticulocyte and that the system is quite complex. Virtually no work has been reported comparing the exchange of phospholipid in reticulocytes, or young and old erythrocytes.

2. MATERIALS AND METHODS

2.1 Materials

[9,10-^{3}H] palmitic acid, (1-^{14}C] linoleic acid and [^{59}Fe] ferrous citrate were obtained from New England Nuclear Corporation, Boston, Mass., [1-^{14}C] linolenic acid from Amersham/Searle Corporation, Chicago, Ill., and $Na_3{}^{32}PO_4$ from Charles E. Frosst and Co., Montreal, Quebec.

Bovine serum albumin (BSA), Fraction V, was purchased from Nutritional Biochemical Co., Cleveland, Ohio. Phospholipids were obtained

from Nutritional Biochemical Co., Serdary Research Laboratories, London 72, Ontario and Supelco Inc., Bellefonte, Pa. Double distilled water, produced by an all glass system, and used in preparation of a buffered saline with glucose solution to be described later was obtained through the courtesy of Dr. Peter Weldon, Department of Physiology, McGill University.

Millipore filters and equipment were purchased from Millipore Corporation, Bedford, Mass.

PPO (2,4-diphenyloxazole) and POPOP [1,4-bis-2-(5-phenyloxazolyl benzene)] were obtained from Packard Instrument Company Inc., Downers Grove, Ill., or New England Nuclear Corporation. The liquid scintillation phosphor cocktail was prepared as follows: 9 gm PPO, 0.3 gm POPOP in 3 litres toluene.

Silica Gel powders were purchased from Machery, Nagel, and Co., Duren, Germany.

General reagents, all of which were reagent grade, and supplies were obtained from Fisher Scientific Co. Ltd., or Canadian Laboratory Supplies Co. Ltd., Montreal, Quebec.

Rabbits were supplied by Canadian Breeding Farms, St. Constant, Quebec.

2.2 Preparation of Labelled Material

2.2.1 In vitro Exchange Experiments

10 mCi of $Na_3{}^{32}PO_4$ were injected intraperitoneally or intravenously into 5-10 lb. male New Zealand White rabbits. Three to four days later the blood was removed by cardiac puncture using 0.25 M sodium citrate as the anticoagulant. The erythrocytes were washed 3-4 times with ice-cold isotonic saline, removing the buffy coat along with the top 10% of the erythrocyte column each time. One volume of packed washed ^{32}P-labelled

erythrocytes was incubated in 5 volumes of Krebs Ringer bicarbonate buffer pH 7.4 (prepared by the method of De Luca and Chen (243) containing 8 mM glucose for 2 hrs. at 37°C, with a gas phase of 95% O_2, 5% CO_2. 30μCi [9,10-^{3}H] palmitate and 10μCi [1-^{14}C] linolenate or [1-^{14}C] linoleate, bound to defatted bovine serum albumin by a modification of the method of Milstein and Driscoll (244), were added per ml of incubated packed cells. The defatted albumin was prepared by the method of Chen (245), dialysed against water for two days with at least two changes of dialysate and lyophilized. After the pre-incubation the erythrocytes were washed at least 3 times with 2% defatted BSA in Krebs-Ringer bicarbonate buffer to remove unincorporated free fatty acids. For experiments involving erythrocyte membranes, "ghosts" were prepared from the labelled cells by successive freezing and thawing in an acetone-dry ice bath and a warm water bath, respectively, three times. The membranes were repeatedly pelleted by centrifugation at 10,800 xg for 10 min. and washed with isotonic saline until the supernatant was nearly colourless. At this point, the membrane pellet was colourless or slightly pink.

Unlabelled serum required for the exchange incubations was obtained after coagulation of blood collected by cardiac puncture from 18 hr. fasted rabbits. Prior to use, this serum was centrifuged at 20,000 xg for 20 min. to remove platelets, leucocytes, and other contaminants. Serum lipoproteins were fractionated by the method of Havel et al (246), using an International B-60 preparative ultracentrifuge with a SB 283 rotor at 100,000 xg at 14°C.

2.2.2 In Vitro Exchange vs. Red Cell Age Experiments

18-20 mCi of $Na_3{}^{32}PO_4$ were injected intravenously into 6-10 lb. rabbits. In one experiment 40 μC ^{59}Fe citrate had been injected a few days

prior to this (247). The blood was collected as already described above either 24 or 40 hrs. after $Na_3{}^{32}PO_4$ injection. The erythrocytes were washed 3-5 times with a buffered saline solution containing glucose (8.10 gm NaCl, 1.22 gm Na_2HPO_4, 0.1937 gm $NaH_2PO_4 \cdot H_2O$, 2.0 gm glucose in 1 litre double-distilled water, pH 7.4, 291 mOsm/kg H_2O) (248) by the method of Prentice and Bishop (247). After each centrifugation all except the last centimetre of supernatant was aspirated and a cotton-tipped stick was rotated at the top of the red cell column. The leucocytes, but not the erythrocytes adhered to the cotton. Thus, the leucocytes were quantitatively removed, without any loss of red cells.

The washed red cells were fractionated according to age by ultracentrifugation on a discontinous BSA density gradient using a slight modification of the method of Piomelli et al (248) to be described later. The erythrocyte fractions and the control populations were pre-incubated with labelled free fatty acids as already described, except for the following modification. 135 µCiof BSA-bound [9,10-^{3}H] palmitate and 45 µCi [1-^{14}C] linoleate were incubated in 17 ml medium per gram of erythrocyte haemoglobin. All other pre-incubation and washing conditions were identical. The unlabelled serum for these experiments was prepared as described in the preceeding section. Haemoglobin was determined by the method of King (249) as modified by Brownstone and Denstedt (250).

2.2.3 In Vivo Exchange Experiments

Erythrocytes of donor rabbits were labelled in the phospholipids with ^{32}P and radioactive fatty acids as already described for the "standard" in vitro exchange experiments. In addition, during the last half hour of the preincubation with the labelled free fatty acids, the cells were labelled with $^{51}CrO_4$ by the method of Mayer et al (251). 2 µCi of $^{51}CrO_4$

per ml preincubation mixture were added to the preincubation suspension. Incorporation of radioactive chromate was stopped five minutes before the end of the preincubation by addition of ascorbic acid (1 mg/ml preincubation mixture). The cells were then separated from the medium and washed with defatted BSA as already described.

2.3 Separation of Red Cells According to Age

Separation of red cells according to age by ultracentrifugation on a discontinuous BSA density gradient was carried out by the method of Piomelli et al (248) with certain modifications. Impurities were first removed from the BSA before preparation of the concentrated solutions. Insoluble particles were separated from BSA by millipore filtration (0.8 μ pore size) of 5-10% solutions and dissolved ions were then removed by dialysis against distilled water for two days with at least two changes of water. The BSA was then lyophilized prior to use. Osmolarity adjustments of the concentrated BSA solutions to 291 mOsm/kg H_2O measured with an Advanced Osmometer Model 600-5 (Advanced Instruments Inc., Newton Highlands, Mass.) was done by addition of solid NaCl, and dilutions were made with 0.92% NaCl (291 mOsm): procedures suggested by Winterbourn and Batt (252). The ultracentrifugation of the gradients at 100,000xg for 50-60 min. at 4°C was prededed by a short centrifugation (10 min.) at 10,000xg to prevent any possible clumping at the first interface of the gradient.

2.4 Exchange Incubation Procedures

2.4.1 In Vitro Exchange Experiments

One volume of labelled erythrocytes or the membranes obtained from one volume of these cells were incubated with 4 volumes of serum or serum lipoprotein solution for 4 hrs. at 37°C under an atmosphere of air. After incubation,

the serum was removed and the cells were then washed 4 times with 2 volumes of cold isotonic saline each time. This washing procedure, necessitated by total volume restrictions of the subsequent lipid extracts, was found to quantitatively separate the serum from the erythrocytes. The washings were extracted together with the serum fraction. In the in vitro exchange vs. erythrocyte age experiments, the exchange incubation conditions were identical except that 17 ml serum per gm erythrocyte haemoglobin was used. The lipids of the cells or membranes and serum were extracted, fractionated, and quantitated. See Appendix for details.

2.4.2 In Vivo Exchange Experiments

15-20 ml of 75% suspension of the quadruply labelled (^{3}H, ^{14}C, ^{32}P, ^{51}Cr) erythrocytes were injected intravenously by the ear vein per recipient rabbit. Thirty minutes later a 5 ml sample of blood was taken for a "zero time" control. Thereafter 5 ml samples were taken at intervals for up to 5 days. The erythrocytes of each sample was quantitated in terms of total mgm haemoglobin and a small aliquot was taken for the counting of the radioactivity due to ^{51}Cr. The rest of the sample was then used for lipid extraction and analysis.

2.5 Analytical Procedures

Cell and serum lipids were extracted by the method of Folch et al (253) and evaporated to dryness under nitrogen. To avoid possible contamination with radioactive free fatty acids, the lipids were redissolved in chloroform and separated into neutral lipids and phospholipids on silicic acid columns, Mallinckrodt 100 mesh, activated overnight at 110-120°C according to the prodedure of Hirsch and Ahrens (254). To all lipid extracts was added 2,6-di-tert butyl-p-cresol (BHT) in a final concentration of 50 mg/100 ml. The antioxidant was also added to the phospholipid fraction

after the silicic acid chromatography. The lipid extracts were manipulated or stored under nitrogen wherever possible. The phospholipids were separated by thin layer chromatography on a binder-free silica gel, MN-Silica Gel N-UV254, using the solvent system of Skipski et al (255) and marked after being visualized with iodine vapour. After de-iodination (upon standing) the bands were scraped off and the phospholipids eluted by the method of Wood et al (256), yielding recoveries of 85%. In some experiemnts, lecithin (PC) was to be further separated into molecular species of differing degrees of unsaturation. PC was separated from other phospholipids as above and visualized with a water spray. The PC was then eluted as above, after the bands had dried and was separated into the molecular species by argentation thin layer chromatography on MN-Silica Gel N (257). These bands were carefully scraped off and the lipid quantitatively eluted with successive 10 ml portions of chloroform-methanol-acetic acid water 100:60:16:8, chloroform-methanol 1:1 and finally methanol. The eluates were washed free of dye and silver ions by the method of Arvidson (257) with a recovery of radioactivity of 70%.

Triple-labelled samples were counted in a Packard Tricarb Model 3003 liquid scintillation spectrometer using PPO-POPOP mixture in toluene as the phosphor. Corrections were made for crossover and quenching. Decay of ^{32}P was corrected back to the time of incubation for the in vitro experiments and to time of injection of radioactive erythrocytes into the recipient rabbits for the in vivo experiments (addurate to less than 6 hrs.). Lipid phosphorus was estimated by a modification of the method of Bartlett (258). The modification entailed the use of 0.6 ml concentrated perchloric acid in the digestion of the samples so that it would be complete in less than 10 min. instead of after several hours.

3. Results

3.1 Behaviour of Phospholipid Exchange with Time

Since the incubation conditions for studying phospholipid exchange *in vitro* have not yet become standard in the literature, a study was undertaken to determine a convenient incubation time.

Exchange of phospholipid from erythrocytes labelled *in vitro* after administration of $Na_3{}^{32}PO_4$ and *in vitro* by incubation for 2 hrs. with BSA-bound [1-^{14}C] linoleate, was observed *in vitro* with time. As shown in Figure 3, exchange of phospholipid labelled by both methods was linear for at least 6 hrs. confirming the findings of Reed (100). Therefore, a 4 hr. incubation period was used as a convenient incubation time for subsequent studies. During these incubations there was no significant change in the quantities of phospholipids present in the erythrocyte, a finding which is consistent with an exchange process, rather than a net transfer.

3.2 Exchange of Phospholipid with Specific Lipoproteins

Serum phospholipids are transported throughout the body by the circulating lipoproteins. Since these lipoproteins are metabolically and structurally heterogeneous, experiments were done to determine whether phospholipid exchange preferentially takes place with any specific lipoprotein or merely depends on the phospholipid content of the incubation medium. Varying quantities of VLDL, LDL, and HDL in separate samples were incubated with erythrocytes which had been labelled *in vivo* after injection with $Na_3{}^{32}PO_4$. The data in Figure 4, which represents the combined results of two experiments, show that the rate of exchange of phospholipid is not influenced by any particular lipoprotein fraction. It is, however, proportional to the concentration of phospholipid in the incubation medium.

Figure 5 is a plot of specific activity of the labelled phospholipid

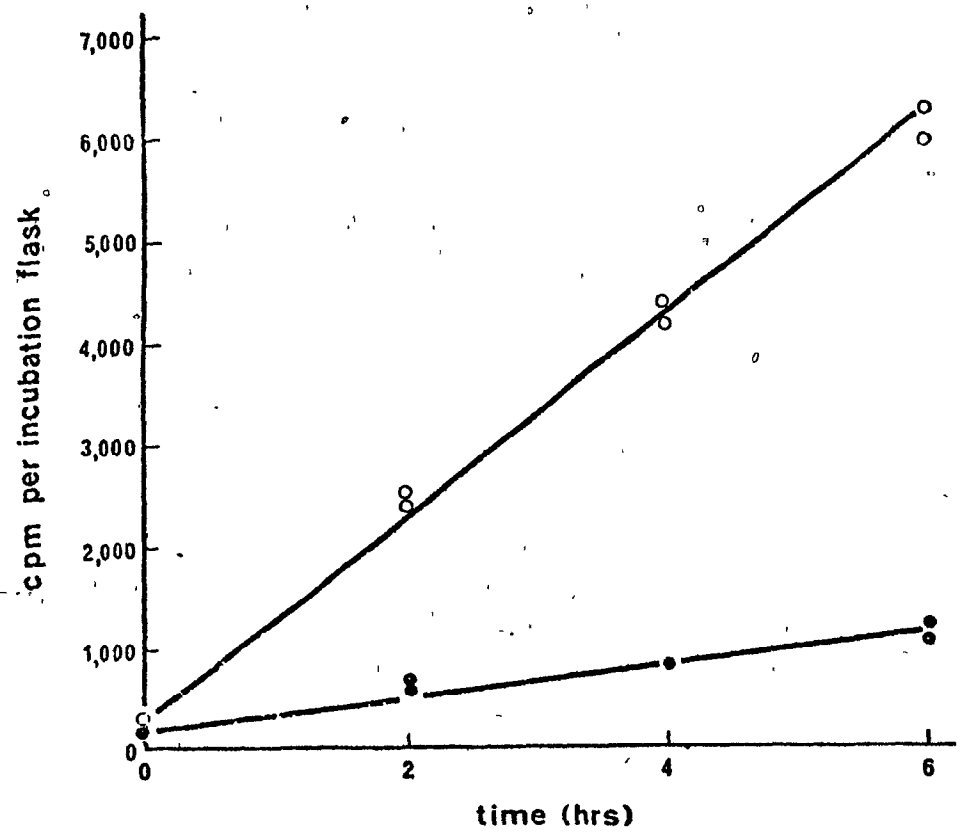


Figure 3: Time course of exchange of labelled phospholipids from erythrocytes into serum. O: ^{32}P, ●: ^{14}C. The data shows values obtained in 2 experiments. Incubation conditions: Washed ^{32}P-labelled erythrocytes were preincubated in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 µCi [1-^{14}C] linoleate bound to albumin, and 8 mM glucose with a hematocrit of 20%. After 2 hrs. the cells were washed with a defatted-albumin containing medium, and divided up into 1 ml aliquots. Each 1 ml aliquot was incubated with 4 ml serum for 0, 2, 4, or 6 hrs. The serum lipids were then extracted and the phospholipids separated from the total lipid extract. The radioactivity of the serum phospholipids of each sample was then counted and the results plotted against time.

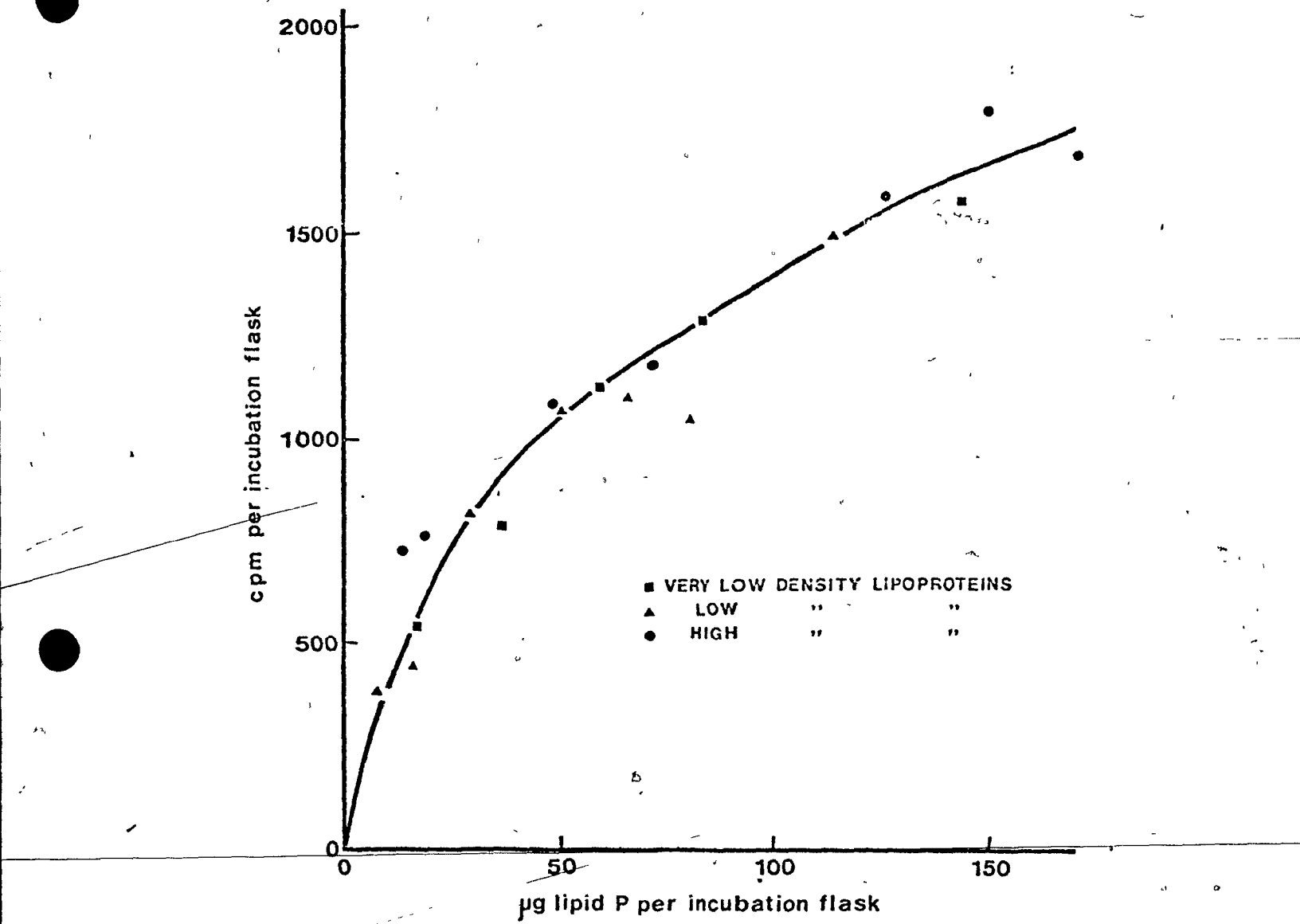


Figure 4: Acquisition of [^{32}P] phospholipid from rabbit erythrocytes by varying amounts of serum lipoproteins. ■: VLDL, Δ: LDL, O: HDL. The data are accumulated values from two separate experiments. Incubation conditions: 1 ml of washed ^{32}P-labelled erythrocytes were incubated with 4 ml of lipoprotein solution for 4 hrs. at 37°C. Lipoprotein solutions consisted of lipoproteins dissolved in Krebs-Ringer bicarbonate buffer, pH 7.4. Lipoprotein phospholipid concentration is expressed as µg lipid P per incubation flask. Control samples consisted of 1 ml cells incubated with 4 ml buffer alone for 4 hrs.

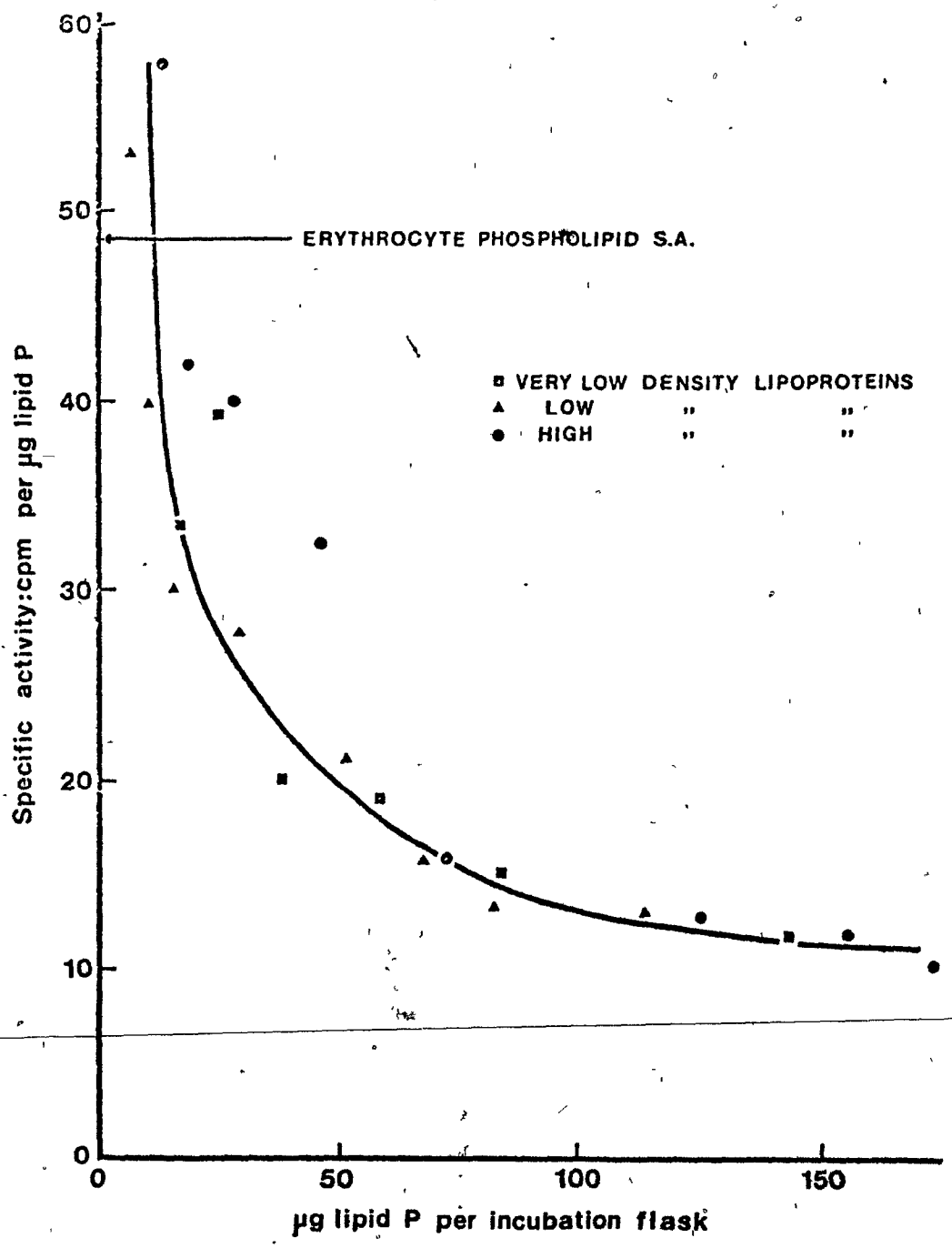


Figure 5: Specific activities of lipoprotein phospholipid ^{32}P after incubation with erythrocytes. ■: VLDL, ▲: LDL, ●: HDL. The data are accumulated values from two separate experiments. Incubation: as in Figure 4.

appearing in the lipoprotein solution after incubation against the lipid phosphorus content of the solution. The specific activity of the labelled phospholipid present in the lipoprotein solution after exchange is extremely high at low medium lipid phosphorus levels but it decreases rapidly as the medium lipid phosphorus concentration increases, until finally, the medium lipid phosphorus concentration has little further effect on the specific activity.

3.3 The Fate of Various Phospholipid Precursors in the Erythrocyte and Serum After Exchange

As a first step in the study of exchange between the rabbit erythrocyte and serum of phospholipid derived from various radioactive fatty acid precursors as well as that labelled with ^{32}P by exchange *in vivo*, a determination was made of the fate of these various labels in the erythrocyte and serum after exchange. Erythrocytes labelled *in vivo* with ^{32}P by exchange were incubated with radioactive free fatty acids and then with serum for 4 hrs. Table V illustrates the distribution of label due to these precursors in the phospholipids of the erythrocytes. Since the amount of label exchanged into the serum over 4 hrs. is quite small, there was little difference in the distribution of erythrocyte radioactivity before and after exchange. It may be observed that regardless of the precursor, most of the label was incorporated into the lecithin fraction of the erythrocyte. These observations are in agreement with previous data (6). The incorporation into PC averaged about 70% for all precursors except palmitate, a larger proportion of which, interestingly, was always incorporated into the phosphatidylethanolamine. No other erythrocyte phospholipids contained a significant proportion of radioactivity. The pattern of incorporation of ^{32}P into the cell was not remarkably different from that of labelled linoleate or linolenate. In all

TABLE V

Distribution of Radioactivity and Lipid Phosphorus in Erythrocytes Following Exchange of Labelled Erythrocyte Phospholipids.

Phospholipid	^{32}P	[^{3}H] Palmitate	[^{14}C] Linoleate	[^{14}C] Linolenate	Lipid P
	% radioactivity				% µgP
Lysolecithin	1	1	1	1	2
Sphingo myelin	8	1	3	2	21
Lecithin	71	54	66	73	39
Phosphatidyl serine +phosphatidyl inositol	3	4	5	4	7
Phosphatidyl ethanolamine	17	41	26	21	29

The data are the means of 3 to 5 experiments with S.E.M. <3% in each of the larger percentages (>20%)

Incubations: Washed ^{32}P-labelled erythrocytes were preincubated with labelled albumin-bound free fatty acids under the following conditions: Hematocrit 20%, Krebs-Ringer bicarbonate buffer, pH 7.4, containing 8 mM glucose and 10 µCi [1-^{14}C] linoleate or [1-^{14}C] linolenate and 30 µCi [9-10-^{3}H] palmitate. After 2 hrs. the cells were washed with a defatted albumin-containing medium and 1 ml of packed cells reincubated with 4 ml serum at 37° for 4 hrs. For the control, 1 ml of packed cells were mixed with 4 ml serum and then separated immediately. See Appendix for further details.

cases, lecithin was the main phospholipid labelled *in vivo* or *in vitro*, but lecithin constitutes only 39% of the total erythrocyte phospholipids, a value which is in agreement with those found in the literature for rabbit erythrocytes (33). Table VI shows that in the serum after exchange, lecithin contained by far the most radioactivity (at least 80%). By contrast serum phospholipid is only 59% lecithin. Apart from a small amount of label in PE containing radioactive palmitate of linoleate, no other serum phospholipid was significantly labelled.

3.4 Comparison of Exchange Into and Out of the Erythrocyte

Although lecithin is the main phospholipid involved in exchange, not all of the erythrocyte lecithin is available for exchange (100,134). Reed has made a quantitative estimate of the exchangeable lecithin present in the human and dog erythrocytes (100). In these calculations, a comparison was made of the fractional exchange rates of lecithin from the erythrocyte (outward exchange) with that of lecithin exchanged into the erythrocyte from the serum (inward exchange). The estimates of the actual amount of erythrocyte lecithin turned over in each direction by exchange were based on the specific activities of the ^{32}P-labelled lecithin found in the serum and cell after exchange in parallel incubations. Since lecithin is neither lost nor gained by the erythrocyte during exchange, the calculated amount of lecithin turned over going in each direction should be identical. In the event that two pools of cellular lecithin exist, one of which does not exchange, the specific activity of the erythrocyte ^{32}P-labelled lecithin used to calculate the fractional turnover will be too low. In the present study, using rabbit erythrocytes and serum, a similar set of experiments were done. Two parallel incubations were set up, one in which ^{32}P-labelled erythrocytes were incubated with unlabelled serum so that exchange of labelled

TABLE VI

Distribution of Radioactivity and Lipid Phosphorus in Serum Following Exchange of Labelled Erythrocyte Phospholipids.

Phospholipid	^{32}P	[^{3}H] Palmitate	[^{14}C] Linoleate	[^{14}C] Linolenate	Lipid P
	% radioactivity				% µgP
Lysolecithin	9	3	2	1	27
Sphingo myelin	8	1	2	1	7
Lecithin	79	82	80	92	59
Phosphatidyl serine + phosphatidyl inositol	2	3	3	2	3
Phosphatidyl ethanolamine	2	11	13	4	3

The data are the mean of 3 to 5 experiments with S.E.M. <3% in each of the larger percentages (>20%).

Incubation Conditions: As in Table V.

lecithin was in the outward direction, and the other in which unlabelled erythrocytes were incubated with ^{32}P-labelled serum, so that exchange of labelled lecithin was in the inward direction. The data enabling calculation of the erythrocyte lecithin pools are shown in Table VII. An average inward exchange rate was determined to be 3.5% while that in the outward direction was 6.5%, giving an average ratio of inward to outward exchange rate of 0.55. This value is similar to that obtained by Reed (100) for human and dog erythrocytes. These data suggest that the value for the specific activity of the erythrocyte lecithin used for the calculation of the outward exchange rate, which was based on the total cellular lecithin content, is too low and only slightly more than half of the erythrocyte lecithin is actually taking part in exchange.

3.5 Participation of Erythrocyte Lecithins Derived by Esterification of Various Free Fatty Acids in Exchange

In light of the finding that only part of the erythrocyte lecithin is available for exchange, it was decided to examine the involvement in exchange with serum lipoproteins, of lecithin formed by the Lands esterification pathway. Exchange of these lecithins was compared to that of the lecithin in the cell labelled with ^{32}P mainly by exchange *in vivo*. The procedure described in the Methods section was followed and the lecithin of the cells and serum were analysed from two points of view. Firstly, the isotopic ratios, $^{3}H/^{32}P$ and $^{14}C/^{32}P$, for each labelled fatty acid precursor, in serum and cell were calculated and compared. In the second approach the rates of exchange for each precursor were calculated and compared. Table VIII summarizes the data and analysis derived from the first point of view. Since [^{32}P] lecithin was incorporated into the erythrocyte *in vivo* over a period of several days by exchange, it represents the pool of cellular lecithin which takes part in

TABLE VII

Comparison of the inward and outward exchange rates of erythrocyte [^{32}P]PC

Expt.	PC content		Inward exchange			Outward exchange			Ratio:
			specific activity		fractional turnover rate	specific activity		fractional turnover rate	
	Serum	Cell	Serum	Cell		Serum	Cell		Inward/Outward
	µg P/flask		cpm/µg P		%	cpm/µg P		%	
1	68	24	3800	140	3.7	55	2700	5.7	.65
2	200	40	3000	100	3.3	17	1200	7.3	.45
Average					3.5			6.5	.55

Incubation conditions:

Outward Exchange - 1 ml ^{32}P-labelled erythrocytes were incubated with 4 ml unlabelled serum for 4 hrs. at 37°C, Cells and serum of control samples were separated immediately after mixing.

Inward Exchange - 1 ml unlabelled cells were incubated with 4 ml ^{32}P-labelled serum for 4 hrs. at 37°C. Cells and serum of control samples were separated immediately after mixing.

TABLE VIII

The Ratio of Fatty Acid (^{14}C or ^{3}H) to ^{32}P in Serum and Cell Lecithin Following Exchange Between Erythrocytes and Serum.

Esterified Fatty Acid	Fatty acid/P ratio *		Ratio **
	A serum	B cells	A/B
Linoleate-^{14}C	3.0	5.7	0.53 ± 05 (10)
Linolenate-^{14}C	3.3	3.0	.94 ± .06 (5)
Palmitate-^{3}H	5.8	6.9	.64 ± 12 (11)

* data from a typical experiment

** mean ± SE of the number of experiments indicated in parenthesis

Labelling and incubation conditions: As in Table V.

exchange. One can calculate the isotopic ratios of radioactive fatty acid-containing lecithin/^{32}P-labelled lecithin for both the serum and cell. If the ratios for the serum are the same as that of the cell as is the case for [^{14}C] linolenate-containing lecithin, then it would appear that virtually all of this lecithin is available for exchange, and when one divides the serum ratio by the cellular ratio the final value is equal to unity. If, however, only part of the fatty acid labelled lecithin in the erythrocyte is available for exchange, then the final ratio of serum ratio to cell ratio will fall below unity. For example, only 53% of the [^{14}C] linoleate-containing lecithin is exchangeable. These ratios were calculated as below.

$$\frac{A_s}{P_s} \Big/ \frac{A_e}{P_e}$$

where A_s = the cpm of acyl-labelled lecithin in serum.

A_e = the cpm of acyl-labelled lecithin in the erythrocyte.

P_s = the cpm of ^{32}P -labelled lecithin in serum.

P_e = the cpm of ^{32}P -labelled lecithin in the erythrocyte.

In the second approach to analysing the isotopic data of the serum and erythrocyte, the rates of exchange for each labelled lecithin were calculated as below:

Fractional exchange rate of fatty acid labelled lecithin = $\frac{A_s}{SA_{e(a)} \cdot Me}$

Fractional exchange rate of ^{32}P-labelled lecithin = $\frac{P_s}{SA_{e(p)} \cdot Me}$

where $SA_{e(a)}$ = specific activity of acyl-labelled erythrocyte lecithin.

$SA_{e(p)}$ = specific activity of ^{32}P-labelled erythrocyte lecithin.

Me = quantity of erythrocyte lecithin.

The fractional rates of exchange for each labelled lecithin are shown in Table IX. About 6% of the ^{32}P-labelled erythrocyte lecithin and [^{14}C] linolenate-containing lecithin were exchanged during the four hour incubation,

TABLE IX

Comparison of the Exchange Acyl-labelled PC and [^{32}P]PC of Erythrocytes.

	Number of Experiments	Fractional exchange rate % / 4 hr.	Relative Exchange rate
[^{32}P]PC	14	6.42 ± .24*	1.0
[^{3}H] palmityl-PC	10	3.90 ± .65	0.64 ± .12
[^{14}C] linoleyl-PC	10	3.45 ± .40	0.53 ± .05
[^{14}C] linolenyl-PC	5	5.96 ± .24	0.94 ± .06

* Mean ± S.E.

Labelling and incubations conditions: As in Table V

but only about 4% of the [^{3}H] palmityl lecithin and 3.5% of the [^{14}C] linoleyl lecithin exchanged.

These rates may be compared by calculating the relative exchange rate. The relative exchange rate is the fractional exchange rate of erythrocyte acyl-labelled lecithin divided by the fractional exchange rate of ^{32}P-labelled erythrocyte lecithin.

$$\text{relative exchange rate} = \frac{A_s}{SA_{e(a)} \cdot Me} \Big/ \frac{P_s}{SA_{e(p)} \cdot Me}$$

$$= \frac{A_s \cdot Me}{A_e} \Big/ \frac{P_s \cdot Me}{P_e}$$

$$= \frac{A_s}{P_s} \Big/ \frac{A_e}{P_e}$$

Thus, in Table IX comparison of the fractional exchange rates of ^{32}P-labelled erythrocyte lecithin with the fatty acid-labelled lecithins shows that while lecithin formed by esterification of [^{14}C] linolenate exchanged at the same rate, [^{14}C] linoleate-containing lecithin and [^{3}H] palmitate-containing lecithin exchanged at rates only 53% and 64%, respectively, of that of ^{32}P-labelled erythrocyte lecithin.

These relative exchange rates are numerically equal to the "ratios of the serum to cell isotope ratios" and therefore taken together these two analyses suggest that, for example, only 53% of the [^{14}C] linoleate-containing lecithin in the rabbit erythrocyte is available for exchange. Since, however, the relative rate of exchange is an easier term to deal with, it alone will be subsequently used.

The observation that 53% of the cellular lecithin produced by esterification of [^{14}C] linoleate is available for exchange corresponds to the size of the exchangeable pool of the total cellular lecithin, which was determined by comparison of the rates of inward and outward exchange of ^{32}P-labelled

lecithin. This would suggest that linoleate esterifies the whole cellular lecithin pool. On the other hand, linoleate, which when esterified, has a relative exchange rate not significantly different from 1.0, and it therefore appears to label only the cellular lecithin pool which can take part in exchange. The relative exchange rate of the lecithin labelled with [^{3}H] palmitate was significantly less than 1.0 [$p<0.05$] but this value showed considerable variation from experiment to experiment. Shohet (158) has noted that the exchange of human erythrocyte lecithin labelled by esterification of [^{14}C] palmitate was slower than that of lecithin labelled with [^{3}H] palmitate which was incorporated by exchange with a lipid emulsion. However, a much longer incubation was necessary in order to show this difference.

3.6 Examination of Possible Sources of Artefacts

The possibility that factors quite unrelated to phospholipid exchange may have been responsible for the differences in the amount of lecithin containing the various radioactive fatty acid residues, appearing in the serum was considered. Several possible such sources of artefacts were examined. It was considered possible that a phospholipase which specifically hydrolysed only certain species of lecithin, might be present in the serum. To test this possibility serum labelled *in vivo* with ^{32}P-labelled phospholipids, by injection of $Na_3{}^{32}PO_4$, was incubated at 37°C for 4 hrs. In another check, erythrocyte lecithin labelled with [^{14}C] linoleate, [^{3}H] palmitate and ^{32}P was emulsified in Krebs-Ringer bicarbonate buffer by sonication on ice and was then incubated with serum for 4 hrs. The results of these checks are found in Table X in Parts A and B respectively. Part A shows that no phospholipid was hydrolysed more than 6% and the hydrolysis of lecithin was only 4%, with the product appearing as lysolecithin. Part B of Table X shows that the ratio of the radioactivity of neither fatty acid label to ^{32}P changed after 4 hrs. of incubation. Thus, it was reasonable to conclude

TABLE X

Effect of 4 hr. Incubation at 37°C on Phospholipid in the Serum

A	^{32}P-labelled Serum
Phospholipid	% Change from Initial Cpm in ^{32}P-labelled serum
Lysolecithin	+ 4%)
Lecithin	- 4%) of Initial Lecithin Cpm
Sphingomyelin	- 2%
Phosphatidyl serine) Phosphatidyl inositol)	- 6%
Phosphatidyl ethanolamine	- 2%

B	Sonicated Labelled Erythrocyte Lecithin
Esterified Fatty Acid	% Change in Ratio of [^{3}H] or [^{14}C] Fatty Acid / ^{32}P
[^{14}C] Linoleate	0
[^{3}H] Palmitate	+ 5%

Incubation conditions: Part A - Serum labelled in vivo with ^{32}P-labelled phospholipids after injection of $Na_3{}^{32}PO_4$ was incubated at 37°C for 4 hrs. The hydrolysis products except for lysolecithin were not detectable with the TLC system used - but only the possible hydrolysis of lecithin was of concern since the lecithin is the major phospholipid exchanged by the rabbit erythrocyte. Part B - Lecithin from 1 ml of erythrocytes labelled with [^{14}C] linoleate, [^{3}H] palmitate, and ^{32}P was emulsified in 0.4 ml Krebs-Ringer bicarbonate buffer by sonication and then incubated with 3.6 ml serum for 4 hrs.

that the difference in the fractional exchange rates between [^{14}C] linoleyl-lecithin or [^{3}H] palmityl-lecithin and [^{32}P] lecithin, and the resulting differences in ^{14}C or ^{3}H/^{32}P ratios between the erythrocyte lecithin and that found in the serum following exchange, was not an artefact due to the presence of phospholipase in the serum. Many investigators (113,134,160) have shown that normal erythrocytes do not contain phospholipase activity.

Lecithin cholesterol acyltransferase in the serum is known to have a high specificity for 2-linoleyl lecithin (200,208). The possibility that this enzyme might be the cause of the difference in the serum to cell isotope ratio was considered unlikely since incubation of erythrocyte lipids in serum for 4 hrs. had no effect on the isotope ratios (Table X). In any case, this possible source of artefact was ruled out by the observation that inactivation of the enzyme by heating the serum at 50°C for about 1 hr. (259) prior to its use in exchange experiments had no effect on the resulting isotope ratios in the serum.

With the elimination of these various factors, two explanations can be offered to account for the apparent existence of two pools of lecithin in the erythrocyte, only one of which is available for exchange with serum lecithin. It is possible, first of all, that only certain specific molecular species of erythrocyte lecithin are exchangeable. Secondly, the physical location of a molecule of lecithin in the membrane (i.e., the inner surface vs. the outer surface of the membrane) or the nature of the binding of the lecithin molecule to the membrane may be responsible for the molecule's ability (or lack of it) to exchange.

3.7 Involvement of Molecular Species of Lecithin in Exchange

The possibility that only certain molecular species of erythrocyte take part in exchange was examined by subdividing the lecithin of the cells and

serum after exchange into their molecular species according to the degree of unsaturation. Table XI illustrates the results of two such experiments. Most of the ^{32}P-labelled lecithin was located in the dienoic species (which may contain some trienoic lecithin as well). The remainder of the [^{32}P] lecithin was found mainly in the saturated and monoenoic fraction. This pattern of labelling among the species of lecithin in the erythrocytes corresponds fairly well to their quantities as shown by Van Golde et al (43). ^{32}P-labelled lecithin appearing in the serum after exchange was also found to be mostly in the dienoic fraction. Somewhat less label appeared in the saturated and monoenoic species of serum [^{32}P] lecithin than was originally in this fraction in the erythrocyte. On the other hand, there was dramatically more [^{32}P] lecithin acquired by the tetraenoic fraction of serum lecithin than was initially in the corresponding species in the cell. It is realized too, that this species of cell lecithin represented a very small percentage of the [^{32}P] cellular lecithin.

Half of the [^{3}H] palmitate was found to be esterified in the dienoic fraction of the cellular lecithin. The saturated and monoenoic species accounted for most of the remainder of the [^{3}H] palmityl cellular lecithin. There was a slight decrease in the distribution of [^{3}H] palmityl lecithin into these species in the serum following exchange. As with the ^{32}P-labelled lecithin, although the tetraenoic fraction of [^{3}H] palmitate-containing lecithin accounted for very little of the lecithin in the erythrocyte, there was a significantly increased amount of this label appearing in the tetraenoic species in the serum. Thus overall, a similar pattern of labelling in both the serum and cell emerged for the ^{32}P-labelled and [^{3}H] palmitate-labelled lecithins.

Upon esterification [^{14}C] linoleate was distributed primarily into the dienoic species of erythrocyte lecithin, with most of the remainder esterifying into tetraenoic lecithins. However, upon exchange into the serum, a

TABLE XI

Distribution of molecular species of labelled erythrocyte PC following exchange with serum.

PC species	^{32}P				[^{3}H]Palmityl-PC				[^{14}C]Linoleyl PC				[^{14}C]Linolenyl PC			
	Expt.1		Expt.2		Expt.1		Expt.2		Expt.1		Expt.2		Expt.1		Expt.2	
	Cells	Serum	Cells	Serum	Cells	Serum	Cells	Serum	Cells	Serum	Cells	Serum	Cells	Serum	Cells	Serum
	% radioactivity															
Saturated and monenoic	22	12	23	11	31	23	29	25	-	-	-	-	-	-	-	-
Dienoic and trienonic	63	57	60	59	52	52	52	42	82	48	79	52	11	9	9	9
Tetraenoic	4	15	5	12	2	9	3	17	14	40	16	37	4	5	5	8
Hexenoic	4	6	5	9	6	6	6	8	1	3	2	4	59	27	57	22
Polyenoic	6	10	7	9	8	10	10	8	3	9	3	3	26	59	29	61
	c.p.m. x 10^{-3}															
Total PC	53	3.1	20	1.2	289	4.3	155	2.8	128	3.2	153	3.5	64	2.8	52	3.2

Incubation conditions: As in Table V

large increase in the percentage of the [^{14}C] linoleyl lecithin in the tetraenoic fraction resulted in an almost even distribution of the linoleate-containing lecithins between the dienoic and tetraenoic fractions. Only a minor amount of radioactivity appeared in the higher unsaturated lecithins.

Six per cent of the [^{32}P] lecithin of the erythrocyte was exchanged in each experiment, as shown in Table XI. The radioactivity found in the tetraenoic species of [^{14}C] linoleyl lecithin was approximately that which would be predicted if one assumed that 6% of the corresponding lecithin species in the erythrocyte took part in exchange during the 4 hrs. of incubation. For example, in experiment 1, there should be about 1300 cpm of [^{14}C] linoleyl lecithin present in the serum, and 1100 cpm were found. Similarly in experiment 2, one should expect about 1500 cpm and 1300 cpm were present. By contrast, the labelled erythrocyte dienoic species was exchanged into the serum at a rate well below 6% and accounts for the lower amount of the total erythrocyte [^{14}C] linoleyl lecithin exchanging into the serum, compared to that of ^{32}P-labelled lecithin. With [^{32}P] lecithin and [^{3}H] palmityl lecithin, exchange of the dienoic species is not as assymmetric with respect to the overall exchange, despite the fact that these species also contain the majority of radioactivity for each label. Therefore, the behavior observed for the dienoic [^{14}C] linoleate-containing lecithins is not characteristic of all dienoic species. The slower exchange rate of the dienoic [^{14}C] linoleyl lecithin is not due to a lack of their corresponding species in serum, since they constitute about 50% of the total rabbit plasma lecithin, almost all of which contains linoleate (43).

Most of the [^{14}C] linolenate esterified by the erythrocyte was found in the hexenoic species of lecithin and the remainder was mainly present in the polyenoic fraction. The hexenoic fraction did not exchange as readily as

the more highly unsaturated species but this was compensated by the rapid exchange of the highly unsaturated lecithins. The differential distribution of radioactive fatty acid precursors in the erythrocyte lecithins of various degrees of unsaturation after esterification, when considered with lecithin exchange patterns which varied according to the precursor, suggest that the site or mechanism of esterification may be an important determinant of the availability of esterified erythrocyte lecithins for exchange.

3.8 Exchange of Lecithin Between Erythrocyte Membrane Preparations and Serum

The possibility that the exchangeable pool of erythrocyte lecithin may be located at the outside surface of the membrane while the non-exchangeable pool is inaccessible to serum lecithin by being situated on the inner surface was entertained. Membranes which were prepared from erythrocytes labelled *in vivo* with ^{32}P and *in vitro* with [^{3}H] palmitate and [^{14}C] linoleate as already described, were incubated with serum for 4 hrs. A parallel incubation with serum of a corresponding aliquot of the intact cells from which these membranes were prepared served as the control.

The distribution of radioactivity among the serum phospholipids was first determined and compared with that of the control. Table XII shows that the exchange of phospholipid between serum and erythrocyte membrane preparations is quite similar to that for the intact cell. The relative amount of lysolecithin exchanged from membranes was increased, but when the overall distribution is considered, this increase was not great. The exchange of ^{32}P-labelled phosphatidylethanolamine was greater for the membrane preparations as well. The percentage of ^{32}P-radioactivity which appeared in the lecithin fraction after exchange from the membranes was somewhat lower than that of the intact cells. However, the relative increase in the amount of label appearing

TABLE XII

Distribution of Radioactivity in Serum After Exchange of Labelled Erythrocyte Membrane Phospholipids.

Phospholipid	[^{3}H] Palmitate		[^{14}C] Linoleate		[^{32}P]	
	Membrane Preparation	Intact Cell	Membrane Preparation	Intact Cell	Membrane Preparation	Intact Cell
Lysolecithin	8	4	4	2	13	7
Sphingomyelin	2	2	2	2	4	8
Lecithin	67	70	80	84	66	82
Phosphatidyl-serine + Phosphatidyl inositol	4	5	3	3	5	1
Phosphatidyl-ethanolamine	19	19	11	9	12	2

Values represent the mean of 3 experiments. In each incubation sample, membranes obtained from 1 ml of packed erythrocytes were incubated with 4 ml serum at 37°C for 4 hrs. These membranes were obtained from erythrocytes labelled as described in Table V. 1 ml aliquots of intact cells from the same erythrocyte preparation were also incubated with 4 ml serum at 37°C for 4 hrs. so that the exchange of labelled lecithin by the membranes could be compared with that by intact cells. "Zero time controls" were run for both membrane and intact cell incubations i.e., membranes and intact cells were separated from the serum of their respctive samples immediately after mixing.

in the lysolecithin and phosphatidylethanolamine fraction accounts for all of the decrease in lecithin radioactivity and it is probable that overall shift in the proportions is due to the increases in the relative quantity of ^{32}P-radioactivity present in the former two fractions rather than a decrease in the latter fraction.

Although not shown, the distribution of radioactivity in the membrane preparations was similar to that of the intact erythrocytes. The small changes in the exchange pattern of radioactivity that did occur were likely due to some alteration done to the membranes during hemolysis. Table XIII gives a comparison of the fractional exchange rates and the relative exchange rates of the erythrocyte membrane preparations versus those of the intact cells. It can be noted that the fractional rates of exchange of [^{32}P] lecithin, [^{14}C] linoleyl-lecithin and [^{3}H] palmityl lecithin between the intact cells serving as controls and the serum was within the range of the larger group shown in Table IX. The fractional exchange rates between erythrocyte ghosts and serum of [^{32}P] lecithin, however, was three fold higher. This large increase in exchange rate is not caused by loss of lipid from the membrane during incubation with serum. For two of the experiments given in Table XIII, the specific activities were determined for the erythrocyte and membrane lecithin before and after the 4 hr. incubation. After incubation the specific activity of the erythrocyte lecithin had decreased by 5 ± 0.8% and that of the "ghost" lecithin had been reduced by 21 ± 4.2%. These calculations were made by using the specific activities of all of the lecithin labels, so that n = 6. The decreases in specific activities correspond to the respective fractional exchange rates found for the intact erythrocytes and the membrane preparations.

When the relative rates of exchange were calculated from the fractional exchange rates, it was found that the values for the exchange of [^{3}H]

TABLE XIII

Exchange of Acyl and ^{32}P-labelled PC in Intact Erythrocytes and Membranes.

	[^{32}P]PC	[^{3}H] Palmitoyl-PC	[^{14}C] Linoleyl-PC
		fractional exchange (% / 4 hr.)	
Intact cell	6.2 ± 0.2	3.6 ± 0.8	3.0 ± 0.1
Membranes	17.1 ± 2.6	22.2 ± 2.6	20.4 ± 2.1
		relative exchange rates	
Intact cells	1.0	0.57 ± 0.16	0.46 ± 0.04
Membranes	1.0	1.27 ± 0.05	1.23 ± 0.08

* Values represent the mean of 3 experiments ± S.E. Each experiment compared the exchange of labelled PC of the intact erythrocytes with membranes prepared from an aliquot of the same cell preparation.

Incubation conditions: As in Table XII. Erythrocyte membranes equivalent to 1 ml of packed cells were added to each incubation flask.

palmityl lecithin and [^{14}C] linoleyl lecithin of the membrane preparations were both significantly greater than unity ($p<0.05$). These values may be compared with the corresponding values obtained for the intact cells, which were about 0.5. Thus, it appears that when erythrocyte ghosts are prepared, not only is the fractional rate of exchange of lecithin greatly increased, but any restrictions in exchange of lecithin derived by esterification of palmitate or linoleate compared to ^{32}P-labelled lecithins is abolished. These results suggest that architecture of the erythrocyte membrane is responsible for these relative differences in exchangeability.

It was thought that these results would be confirmed by exchange experiments in which inside-out vesicles were incubated with serum. These vesicles would be expected to exchange very little if any [^{32}P] lecithin with the serum, since labelling occurs by exchange (100) and PC trans-bilayer migration is slight (53). The method of Steck et al (260) was used in an attempt to prepare the inside-out vesicles. The pattern of separation of the inside-out and right-side out vesicles after ultracentrifugation on the dextran density gradient was clear and identical to that obtained by the authors. Steck et al (260) showed that inside-out vesicles prepared from human erythrocytes are resistant to the action of sialidase, but right-side out vesicles are not. However, when the susceptibility of the rabbit inside-out and right-side out vesicles were compared, they were found to be very similar. This suggested that the inside-out vesicles were quite leaky, perhaps reflecting a basic species difference in the resealing ability of the membranes. When the vesicle preparations were incubated with serum, the results were similar to those obtained with ordinary erythrocyte ghosts (Table XIII).

While this project was in progress, a patient who was being treated for polycythemia by injection of $Na_3{}^{32}PO_4$, at the Royal Victoria Hospital, Montreal, became available for donation of a sample of blood. An exchange

experiment using his erythrocytes was attempted, but the erythrocyte lecithin was not labelled heavily enough with ^{32}P, however, to provide a clear indication of the [^{14}C] linoleyl/[^{32}P] ratios in serum lecithin after exchange.

3.9 Exchange of Lecithin by Young and Old Erythrocytes

As erythrocytes age *in vivo*, membrane lipid is lost (221,222) and eventually membrane alterations may occur prior to destruction in the spleen (229). In view of these facts and the results which suggest that membrane architecture is important in the exchange of erythrocyte lecithin with serum, experiments were performed which compared lecithin exchange in young and old erythrocytes. Cells labelled *in vivo* after injection of $Na_3\ ^{32}PO_4$, and separated according to age on a BSA density gradient, were labelled *in vitro* by esterification of [^{3}H] palmitate and [^{14}C] linoleate and incubated with serum for 4 hrs. A comparison of the distribution of radioactivity due to the various precursors incorporated into the young and old red cells is shown in Table XIV. Esterification of [^{3}H] palmitate into the cephalin fraction was greater in the old red cells than in the young cells. The pattern of incorporation of [^{14}C] linoleate among the cellular phospholipids did not vary significantly with the age of the cell. This observation is in agreement with previous findings reported by this laboratory for young and old human erythrocytes (6) and for rabbit reticulocytes and mature rabbit erythrocytes (7). More radioactivity due to ^{32}P was found in the cephalin fraction of young red cells than in old red cells. The differences in the behavior of the incorporation of these various precursors as related to the age of the red cell is unclear.

Although the pattern of incorporation of the various precursors into red cell phospholipid may vary with the age of the red cell, there appears to be no age-related variation in the pattern of exchange of the cellular phos-

TABLE XIV

% Distribution of Radioactivity in Red Cells after Exchange of labelled Phospholipids of Young and Old Red Cells

Phospholipid	[^{3}H] Palmitate				[^{14}C] Linoleate				^{32}P			
	Expt. 1		Expt. 2		Expt. 1		Expt. 2		Expt.1		Expt.2	
	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old
Lysolecithin	1	1	1	1	1	1	1	1	1	2	1	1
Sphingomyelin	2	3	4	3	2	3	3	3	6	8	5	4
Lecithin	70	43	71	60	78	69	75	77	68	76	66	75
Cephalin	27	53	25	36	19	27	21	19	25	14	28	20

Incubation conditions: As in Table V.

pholipid into serum. Table XV illustrates the distribution of radioactivity found in serum phospholipids after exchange incubations with young and old red cells. For each of [^{3}H] palmitate, [^{14}C] linoleate, and ^{32}P the exchange response with respect to age was the same. There was no significant change in the pattern of phospholipid exchange with the age of the red cell.

Since it has been considered that the changes in the erythrocyte membrane which occur with age might have an effect on lecithin exchange despite the fact that the overall exchange pattern might not be affected, the fractional exchange rates and the relative exchange rates of [^{3}H] palmityl-, [^{14}C] linoleyl-, and [^{32}P] lecithin and old red cells were examined. The results are given in Table XVI. The unfractionated red cell population consisted of erythrocyte aliquots taken from the same sample of red cells prior to partitioning according to age. These cells were then pre-incubated with the radioactive free fatty acids and then incubated with serum under the same conditions as the young and old cell fractions. The fractional rates of exchange of [^{32}P]-, [^{3}H] palmityl-, and [^{14}C] linoleyl-lecithins in the young cell fraction (the youngest 10% of the whole population) were lower than the respective values of the unfractionated whole population but were not statistically significantly different from these values. Further, the fractional exchange rates of both populations of erythrocytes fell within the range found in the larger series (Table IX).

On the other hand the oldest 10% of the red cell population exchanged labelled lecithins with serum at fractional rates which were about double that of the unfractionated population. Despite this large increase in the fractional rates of exchange of [^{3}H] palmityl-, [^{14}C] linoleyl-, and [^{32}P] lecithin with red cell age, the rates of exchange of the acyl-labelled lecithins relative to that of [^{32}P] lecithin for all cell population remained at about 0.5.

TABLE XV

Distribution of Radioactivity in Serum after Exchange of Labelled Phospholipids in Young and Old Red Cells.

Phospholipid	[^{3}H]-Palmitate				[^{14}C]-Linoleate				^{32}P			
	Expt.1.		Expt.2		Expt.1		Expt.2		Expt. 1		Expt. 2	
	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old
Lysolecithin	10	7	7	2	6	4	1	1	10	8	7	5
Sphingomyelin	5	1	2	2	4	1	1	2	8	5	4	5
Lecithin	59	68	83	76	81	80	97	93	79	80	87	88
Cephalin	26	24	8	20	9	15	1	4	3	7	7	2

Incubation conditions as in Table v

TABLE XVI

Comparison of exchange of acyl- and ^{32}P-labelled PC in young and aged erythrocytes

Cell Population	Fractional Exchange Rate (%/4h)			Relative Exchange Rate ([^{32}P]PC = 1.0)	
	[^{32}P]PC	[^{3}H]palmityl-PC	[^{14}C]linoleyl-PC	[^{3}H]palmityl-PC	[^{14}C]linoleyl-PC
Young	7.1 ± 0.3	3.0 ± 0.2	3.1 ± 0.3	0.42 ± 0.04	0.43 ± 0.06
Old	13.5 ± 1.5	6.3 ± 0.7	7.8 ± 1.9	0.47 ± 0.02	0.55 ± 0.08
Unfractionated	8.9 ± 0.4	3.6 ± 0.8	3.2 ± 0.4	0.39 ± 0.09	0.36 ± 0.04

The values represent the mean of four determinations ± S.E. Incubation conditions as in Table V

3.10 Exchange of Lecithin Between Erythrocytes and Serum in vivo

A study was conducted of the exchange of lecithin between erythrocytes and serum *in vivo*. Erythrocytes were labelled *in vivo* with ^{32}P after injections of $Na_3{}^{32}PO_4$ and *in vitro* by esterification of [^{3}H] palmitate and [^{14}C] linoleate. The cells were additionally labelled with $^{51}CrO_4$ in order to correct for loss of radioactivity due to the usual disappearance of cells from the circulation. Decay curves of the isotopes from the erythrocytes are shown in Figure 6. The disappearance of ^{51}Cr was slow and gradual over the period of the experiment indicating a normal rate of removal of erythrocytes from the circulatory system. The loss of radioactivity of [^{32}P] lecithin, [^{3}H] palmityl lecithin and [^{14}C] linoleyl lecithin from the circulation was more rapid.

When correction is made for the normal removal of erythrocytes from the circulation, the decay curves showing the disappearance of [^{32}P] lecithin, [^{3}H] palmityl lecithin, and [^{14}C] linoleyl lecithin due to exchange alone, may be drawn with the aid of a standard linear least squares analysis and the half-life values of the fractional rates of exchange of each labelled lecithin calculated. These values are shown in Table XVII. The actual fractional exchange rates varied considerably from one experiment to another and were much lower than the corresponding *in vitro* values. When the relative rates of lecithin exchange *in vivo* were calculated, there was a remarkable consistency of results among the experiments. The average relative rate of [^{3}H] palmityl lecithin was not different from that of [^{32}P] lecithin. However, relative rate of [^{14}C] linoleyl-lecithin (0.90) was significantly lower than that of [^{32}P] lecithin.

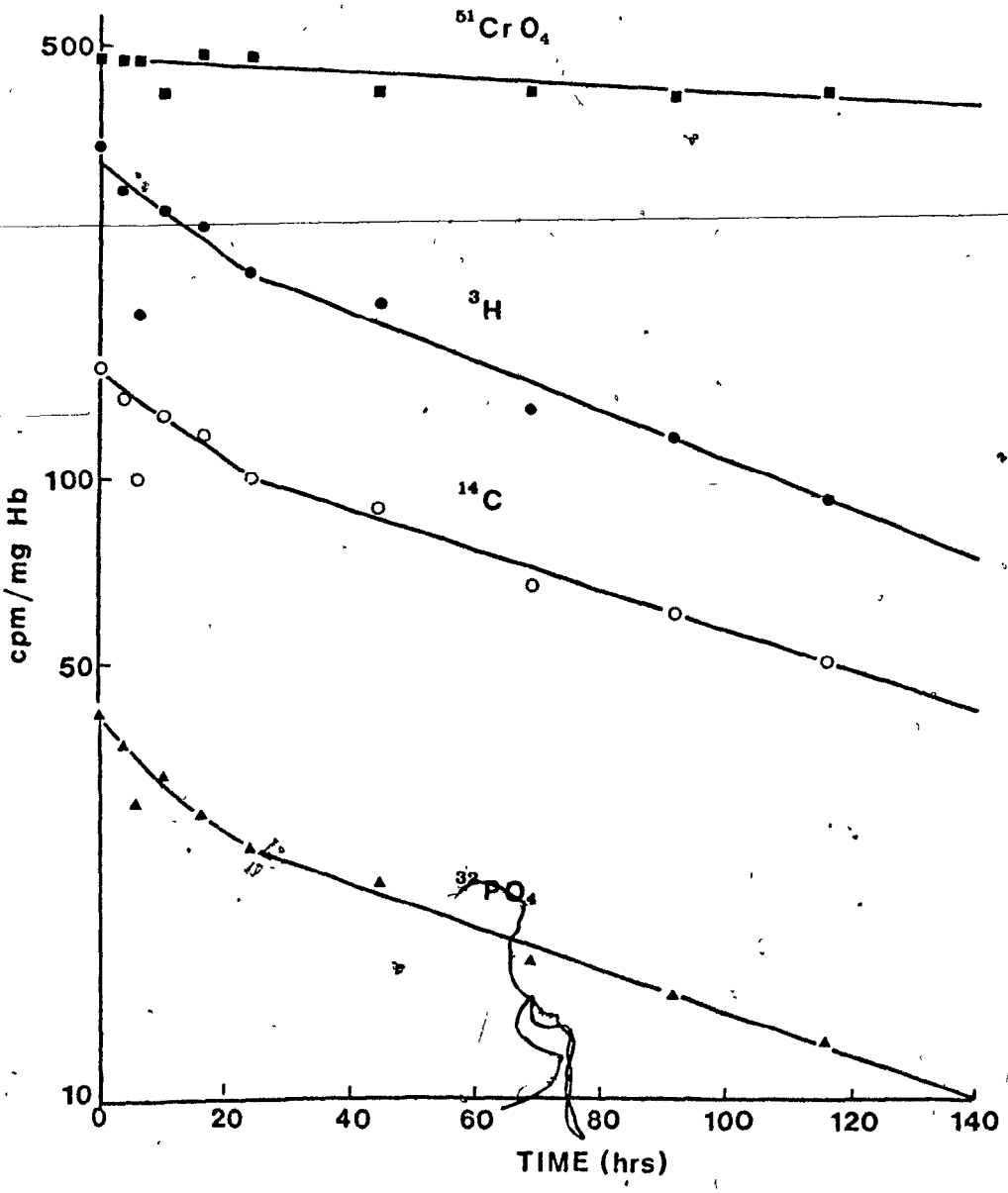


Figure 6: Labelled Lecithin Decay in vivo.

■ $^{51}CrO_4$, ●: [^{3}H] palmityl lecithin, O: [^{14}C] linoleyl lecithin ▲: ^{32}P-labelled lecithin. Erythrocyte lecithins were labelled as in Figure 5 and the cells were additionally labelled with $^{51}CrO_4$ near the end of the in vitro esterification. The erythrocytes were injected into a rabbit and 5 ml samples taken at given time intervals.

TABLE XVII

Relative Rates of in vivo Exchange of Lecithins

Containing [^{3}H] Palmitate and [^{14}C] Linoleate

Labelled Precursor	Exchange Rate % / hr *			Mean ± S.E.
	Expt.1	Expt.2	Expt.3	
[^{3}H] Palmitate	.97	.70	.82	
[^{14}C] Linoleate	.91	.56	.71	
$^{32}PO_4$	.99	.64	.80	
	Ratio of Exchange Rates			
$^{3}H/^{32}P$	.98	1.08	1.02	1.03 ± .03
$^{14}C/^{32}P$	.92	.88	.89	0.90 ± .01

* corrected for erythrocytes removed from the circulation

4. DISCUSSION

In considering exchange experiments, it was assumed that ^{32}P-labelled phospholipids are incorporated into the circulating erythrocyte *in vivo* primarily by exchange. During erythropoesis some [^{32}P] is probably incorporated by other pathways into the phospholipids of developing red cells, but this accounts for only a small percentage of the total phospholipids in the whole erythrocyte population. Additionally, the rabbits were exsanquinated only 3-4 days after injection of $Na_3{}^{32}PO_4$. Therefore, since the rabbit erythrocyte is generally considered to have a life span of 65 days in the circulation, the 3-4 day time period represents a maximum possible contamination of 5-6% of the total circulating population by newly released red cells. When these labelled erythrocytes were collected they were washed by aspiration of leucocytes along with the top 10% of the red cell column at least three times in succession. Thus, the reticulocyte population and to some extent the young mature red cell population were greatly reduced. Since the population of erythrocytes which was used for the exchange incubation consisted entirely of essentially mature cells, the [^{32}P] phospholipid radioactivity present in these cells was incorporated almost completely by exchange. A small amount of the [^{32}P] phospholipid may have been incorporated by esterification of [^{32}P] lysolecithin. However, quantitatively the [^{32}P] lecithin derived from this source would not amount to much since serum [^{32}P] lysolecithin is present in serum in concentrations of less than 10 per cent of the ^{32}P-labelled serum lecithin levels and the rate of fatty acid esterification and phospholipid exchange are roughly the same (158). In the erythrocyte, [^{32}P] lecithin accounts for over 70% of the labelled phospholipid. This roughly corresponds to the distribution of labelled lecithin among the serum phospholipids, but lecithin accounts for only about 40% of the total erythro-

cyte phospholipid phosphorus. Therefore essentially all of the labelled lecithin of the erythrocyte appears to be incorporated from the serum by exchange. Erythrocytes can be labelled by exchange of phospholipids *in vitro* with labelled serum, isolated lipoproteins or with emulsions of lipids obtained from either plasma (100), or erythrocytes (158). In this study however, the *in vivo* method of labelling was used because phospholipid exchange is a slow process (about 1%/hr (100,133), and a lengthy *in vitro* incubation would be required to obtain a sufficient degree of labelling of the cells). Erythrocytes labelled *in vivo* would be strongly labelled and be closer to isotopic equilibrium with labelled phospholipids of the serum. Thus, the distribution of the label would correspond much better to that of the plasma and the erythrocyte would become much more highly labelled *in vivo* than in a relatively short *in vitro* incubation. From a practical standpoint too, is the fact that the erythrocyte preparations would be subjected to much less *in vitro* and manipulative abuse which, in certain experiments, was critical for the preventation of significant haemolysis.

Early in this study, a time curve of phospholipid exchange was performed to determine a convenient incubation period. The linearity of the response was not surprising in view of the fact that phospholipid exchange is a rather slow process, having a turnover time which is measured in days.

It was originally thought that because of the heterogeneity of the lipoprotein classes of the serum, one particular lipoprotein might serve as a reservoir of phospholipid for exchange with the rabbit erythrocyte, such as is the case for rat serum lysolecithin (139). However, the data of Figures 4 and 5 indicate that lipoprotein heterogenity is quite unrelated to phospholipid exchange and that exchange rate is dependent on the quantity of serum lipoprotein phospholipid present in the medium. At low levels of

medium phospholipid the specific activity of the serum phospholipid (Figure 5) is quite high, approaching that of the erythrocyte itself, but this specific activity decreases rapidly to a fairly constant level as the medium phospholipid concentration increases. This suggests that a very small pool of rapidly exchanging cellular phospholipid may exist which is detectable only when the phospholipid concentration of the medium is very low. Thus, a small amount of heterogeneity in the exchangeable pool of cellular phospholipid may exist. However, under normal incubation and physiological conditions, in which the serum phospholipid levels are relatively high, the small rapidly exchanging pool likely represents only a minor portion of the total exchangeable lecithin pool.

In order to study the participation in exchange of cellular phospholipids derived by esterification of various fatty acids, erythrocytes were first pre-incubated with radioactive free fatty acids. Radioactive linoleate, linolenate, and palmitate were all primarily esterified into lecithin. A smaller percentage of these precursors was also found in the phosphatidylethanolamine fraction of the cellular phospholipids. These data (Table V) are in agreement with previous findings (6,96) and provide further evidence which suggest that the esterification pathway primarily uses lysolecithin as a substrate, but also, to some extent uses lysophosphatidylethanolamine as well. The variation in the relative amounts of fatty acid precursor incorporated into phosphatidylethanolamine likely reflects a fatty acid specificity of the esterification reaction.

After erythrocytes labelled with ^{32}P and radioactivity from the various free fatty acid precursors were incubated with serum, 80% of phospholipid radioactivity was usually present in the lecithin of the serum. With perhaps the exception of the appearance of some [^{3}H] palmityl- and

[^{14}C] linoleyl lecithin in the phosphatidylethanolamine fraction, and a very small amount of [^{32}P] in sphingomyelin and lysolecithin, no other phospholipids of the serum were significantly labelled after exchange. This is in interesting contrast to the pattern of phospholipid exchange in the rat. Rabbit serum, like rat serum, contains a relatively high amount of lysolecithin, but unlike in the rat erythrocyte (139), the rabbit red cell does not exchange a quantitatively large amount of lysolecithin. The reason for this difference is unclear.

In the present study, comparison of the rates of exchange in the inward and outward directions relative to the erythrocyte showed that, in confirmation of the observations of Reed in dog and human erythrocytes (100), not all of the rabbit erythrocyte lecithin is available for exchange. Comparison of the size of the exchangeable pool, estimated at 55% of the total cellular lecithin, (Table VII) with the rates of exchange of lecithins labelled by esterification of [^{3}H] palmitate and [^{14}C] linoleate relative to the rates of lecithins labelled by exchange, suggested that these two fatty acids may be esterified throughout the cellular lecithin pool. On the other hand, linolenate, when esterified into lecithin, exchanged at a rate not significantly different from that of lecithin labelled by exchange. Therefore, linolenate may be esterified only into the lecithin pool which is available for exchange with serum.

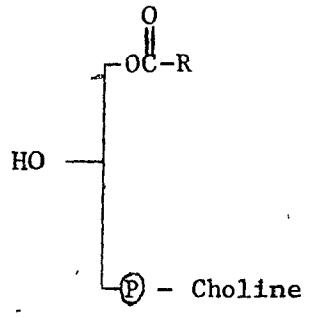
The observation that the fractional rate of exchange of erythrocyte lecithin labelled by esterification with palmitate or linoleate differs from that labelled with ^{32}P or [^{14}C] linolenate, prompted the examination of the erythrocyte-serum system for possible sources of artefacts. It was found that neither lecithin cholesterol acyltransferase (LCAT) activity, nor possible phospholipase A activity in the serum was involved. Phospholipase A_2

activity in the normal untreated intact erythrocyte has not been demonstrated, despite repeated attempts (113,160).

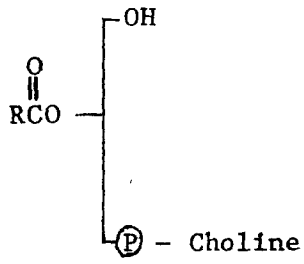
The possibility that a preferential exchange of certain specific molecular species of lecithin might account for the differences seen in the fractional exchange rates of [^{3}H] palmityl- and [^{14}C] linoleyl lecithin compared to that of [^{14}C] linolenyl and [^{32}P] lecithin was considered. Examination of the distribution of molecular species of [^{32}P] lecithin in the erythrocytes showed that it corresponded to the quantities of these species reported by Van Golde (43) for rabbit plasma. This would suggest that lecithin exchange is non-specific with respect to the molecular species. [^{3}H] palmityl lecithin appeared to show no dramatic preferential exchange in any given molecular species of lecithin, and yet the overall rate of exchange was much lower than that of [^{32}P] lecithin. The observation that there apparently is a pool of dienoic [^{14}C] linoleyl lecithin which is not available for exchange is not unexpected since the molecular species accounts for 80% of the total cellular [^{14}C] linoleyl lecithin. The other molecular species of this labelled lecithin are entirely exchangeable. Thus erythrocyte lecithin produced by esterification of [^{14}C] linoleate appears to exhibit some specificity of exchange with regard to molecular species. With [^{14}C] linolenyl erythrocyte lecithins the decreased rate of exchange of the hexenoic species was compensated by the rapid exchange of the polyenoic species so that the overall rate of exchange is similar to that of [^{32}P] lecithin.

It is of interest to note that in the case of [^{3}H] palmitate and [^{14}C] linolenate, the fatty acid was esterified into molecular species which are more unsaturated than that which would be accounted for by an esterification of the free fatty acid to the 2-acyl position. With the other substrate in the esterification reaction, lysolecithin, two chemical species may

exist: the 1-acyl isomer and the 2-acyl isomer.



1 acyl isomer



2 acyl isomer

The fatty acid moiety of the 1-acyl isomer is generally considered to be saturated and the acyl moiety of the 2-acyl isomer unsaturated. Thus some 2-acyl lysolecithin may be taking part in esterification. In contrast to the behaviour of erythrocyte of other species, notably man (95), rabbit red cells have been found by Mulder and van Deenen (96) to esterify palmitate primarily to the 1-position of lysolecithin. Only 9% was found at the 2-acyl position. Such an observation explains the presence of most of the [^{3}H] palmitate radioactivity in the unsaturated molecular species. On the other hand rabbit erythrocytes have been observed to esterify 92% of the incorporated linoleate to the 2 position (96) and this is in agreement with the present findings that this fatty acid was found predominantly in the dienoic species of erythrocyte lecithin. On this basis it is reasonable to suggest that linolenate was esterified predominantly with 2-acyl lysolecithin. The observed differences in the mechanism or the site of esterification of linoleate compared to the other fatty acids, or the presence of specific lysolecithins might explain the faster rate of esterification of linoleate than of palmitate or linolenate into lecithin by intact erythrocytes (96). These observations might also provide insight into the differences in the rate of linoleate esterification of intact erythrocytes of different mammalian species, rate differences which are abolished when hemolysates are used instead (96).

Preparation of haemoglobin-free erythrocyte membranes by definition requires a breaking of the membrane of the intact cell to be successful in the release of the soluble intracellular contents. Of necessity, stress is exerted on the erythrocyte membrane during this process and some alterations may occur (261). Nevertheless the erythrocyte ghost is quite useful and has been widely used in the study of erythrocyte membrane properties. For this study, erythrocytes which had been previously labelled with ^{32}P *in vivo* and with [^{3}H]- and [^{14}C]-fatty acids by esterification *in vitro* were frozen-thawed in the absence of Ca^{++} and Mg^{++} to prevent any possible resealing (262). Examination of the pattern of exchange of labelled phospholipids into serum shows that while some alterations did occur, the distribution of radioactivity, and therefore the overall exchange of phospholipids for erythrocyte membranes is similar to that of the intact cell. This finding is not too surprising considering that the pattern of exchange is likely dependent to a large degree on the distribution of serum phospholipid. The observation that erythrocyte membranes exchange lecithin at an increased rate compared to the intact cell does not have any proven explanation at present. It is possible that there was some alteration in membrane structure or conformation which exposed more membrane lecithin to the serum lecithin. However, this increase in exchange rate is not due to an alteration which involves the non-specific loss of membrane lipid, such as occurs in spherocytosis (263), since the decrease in specific activity of the lecithin of either the intact erythrocyte or the membrane preparation takes place parallel to the fractional exchange rates.

As well as an increase in the fractional exchange rates of the lecithin of the erythrocyte membrane preparations compared to the intact cell, a change in fractional exchange rates of lecithins labelled by esterification of free fatty acids relative to that of lecithin labelled by exchange, took

place. This change may be a result of the location of esterification in the membrane. It is possible that some of the lecithin was esterified in an area inaccessible to the serum, such as the inner surface or the interior of the erythrocyte membrane. So long as the cell remained intact the lecithin produced in this area was not available for exchange. Bretscher has suggested that little exchange or "flip-flop" of phospholipid between the two sides of the bilayer of the erythrocyte membrane occurs. Thus it seems reasonable that when erythrocyte ghosts are prepared, the inner surface of the membrane is exposed and all of the erythrocyte lecithin becomes available for exchange. Therefore the rates of exchange of [^{3}H] palmityl and [^{14}C] linoleyl lecithin relative to that of [^{32}P] lecithin, which were about 0.6 in the intact cell, now increase to about 1.25. The lecithin on the internal surface of the erythrocyte membrane was labelled by esterification with [^{3}H] palmitate, and [^{14}C] linoleate but not by exchange with [^{32}P] lecithin. Thus, hemolysis led to no further exposure of [^{32}P] lecithins, only the lecithins containing the labelled fatty acids. van Golde et al (43) have speculated that some lecithin molecules may remain bound to the erythrocyte membrane during the entire lifespan of the cell while others are free to exchange and that these differences may be attributable to the location of the molecules or their chemical structure. The present data provides experimental evidence which tends to support this speculation. It has been suggested by Gordeski and Marinetti (66) that phosphatidylserine is present almost completely on the interior surface of the erythrocyte membrane and by Verkleij et al (68) that phosphatidylethanolamine may be similarly though not as entirely oriented at the inner side of the membrane bilayer. It is possible that certain species of lecithin and lysolecithin are also asymmetrically distributed in the membrane. Thus a large amount of the saturated 1-acyl isomer of lysolecithin with which linoleate esterifies, may be located at the inner part of the

membrane and the unsaturated 2-acyl isomer which becomes esterified with linolenate may be present at the outer surface of the membrane.

In confirmation of previous studies done in this laboratory with human and rabbit red cells, the present data showed that the distribution of linoleate among the cellular phospholipids, incorporated by esterification by the erythrocyte does not vary with age. The proportion of palmitate esterified into phosphatidylethanolamine increased with age while the proportion of ^{32}P-labelled lecithin incorporated by exchange into phosphatidylethanolamine was decreased in old cells. The preliminary nature of these observations does not enable an explanation of them and a more detailed study would be necessary to clarify these points.

The finding that the pattern of exchange of [^{3}H] palmitate, [^{14}C] linoleate-, and ^{32}P-labelled phospholipids does not seem to be affected by the age of the red cell is perhaps not too surprising considering that the loss of lipid from the plasma membrane which is associated with erythrocyte aging, is non-specific (221,222) and does not affect the intactness of the membrane. The observation that the relative rates of exchange of [^{3}H] palmityl-lecithin and [^{14}C] linoleyl-lecithin compared to [^{32}P] lecithin do not change with the age of the erythrocyte is also consistent with the maintenance of cellular integrity despite age-related lipid loss, and supports the concept that the unavailability of a portion of the lecithin for exchange depends on the intactness of the cell.

Finally, although the relative rates of exchange of the lecithins, and hence the pools of non-exchangeable lecithin are not disturbed by the red cell age, the fact that the actual fractional exchange rates of [^{3}H] palmityl-, [^{14}C] linoleyl- and [^{32}P]-lecithin increased with the age of the red cell suggests that some changes are occurring in the erythrocytes' membrane.

The values for the fractional lecithin exchange rates of young red cells may have been lower than that of old cells partly due to incorporation of $^{32}PO_4$ into reticulocyte phospholipid and due possibly to some extent to uptake of $^{32}PO_4$ during erythropoesis. The latter contribution was minimized however, since for these experiments, the ^{32}P-labelled erythrocytes were collected as soon as 24 hrs. after injection of $Na_3{}^{32}PO_4$. Compared to that of lecithin exchange, the amount of ^{32}P label incorporated during de novo phospholipid synthesis in the reticulocyte is likely quite small. Thus, the increase in the rate of lecithin exchange with increasing red cell age appears to be primarily associated with the changes in the membrane which are known to occur as the red cell matures and ages.

A study of phospholipid exchange in vivo was undertaken to determine if characteristics similar to those observed in vitro could be detected. There was great variation observed in the fractional rate of lecithin exchange and this may have reflected variation in the serum phospholipid levels from animal to animal. Figure 4 shows that the rate of exchange in vitro does depend on the phospholipid concentration of the medium. The observation that the rate of lecithin exchange in vivo was much lower than that observed in vitro (Table IX) may be due to the fact that the hematocrit used for exchange experiments in vitro was 20% while the hematocrit in vivo is normally in the range of 35-45% (20). Thus, in the in vitro conditions there is relatively more extracellular lecithin present in the medium for exchange with the labelled lecithin of the erythrocyte and the radioactive lecithin of the erythrocyte is therefore removed from the cell at a greater rate than that in vivo.

The average relative exchange rates for [^{3}H] palmityl- and [^{14}C] linoleyl-lecithin in vivo were different from the corresponding values observed

in vitro. *In vitro* the relative exchange rates for [^{3}H] palmityl- and [^{14}C] linoleyl-lecithin were 0.64 and 0.53 while the values *in vivo* were 1.0 and 0.90 respectively. The reasons for the discrepancy between the *in vitro* and *in vivo* results are unclear although there are some possibilities. The determination of the rate of disappearance of label from the erythrocyte *in vivo* may be less precise and sensitive than the observation of the rate of appearance of label in the serum *in vitro*. A more likely explanation however is the fact that the *in vitro* system is static and no changes in the serum phospholipid composition are possible. The *in vivo* experiment on the other hand took place over a period of 5 days and great changes in the composition of the serum lecithin were possible. Thus, alterations of the rate of exchange of [^{3}H] palmityl- and [^{14}C] linoleyl-lecithin may have occurred.

5. SUMMARY

1. Exchange of radioactive phospholipid between the rabbit erythrocyte and serum at 37°C *in vitro* was linear with time for at least 6 hrs.

2. The rate of phospholipid exchange was found to depend on the quantity of phospholipid present in the medium. No particular class of serum lipoprotein preferentially took part in exchange.

3. When rabbit erythrocytes were labelled with [^{32}P] phospholipids by exchange *in vivo* and with [^{14}C] linoleate or [^{14}C] linolenate by esterification *in vitro*, about 70% of each of these precursors were incorporated into lecithin. For [^{3}H] palmitate, a somewhat smaller percentage (54%) was found in lecithin, due to an increased incorporation in phosphatidylethanolamine.

4. [^{14}C] linoleate and [^{3}H] palmitate were each esterified primarily into the dienoic species of the erythrocyte lecithin and [^{14}C] linolenate mainly into molecular species with more than four double bonds. ^{32}P was incorporated by exchange mainly into the dienoic erythrocyte lecithins.

5. After *in vitro* exchange with unlabelled serum at least 80% of the radioactivity found in the serum was present in lecithin. Apart from a smaller amount of [^{3}H] palmitate and [^{14}C] linoleate in serum phosphatidylethanolamine no other serum phospholipid was significantly labelled.

6. The radioactivity of [^{32}P] lecithins and [^{3}H] palmityl-lecithins found in serum after exchange were distributed among the various molecular species in patterns similar to that of the erythrocyte lecithins. The dienoic species of [^{14}C] linoleyl erythrocyte lecithin was exchanged at a reduced rate. The decreased rate of hexenoic [^{14}C] linolenyl erythrocyte lecithin exchange was compensated by the more rapid exchange of the polyenoic [^{14}C] linolenyl lecithin.

7. Analysis of the inward and outward exchange rates of [^{32}P] lecithin revealed that only about 55% of the total cellular lecithin of the rabbit erythrocyte was available for exchange.

8. The fractional exchange rates of [^{3}H] palmityl- and [^{14}C] linoleyl-lecithin were 64% and 53% respectively of that of [^{32}P] lecithin while that of [^{14}C] linolenyl lecithin was not significantly different from [^{32}P] lecithin.

9. Serum phospholipase and lecithin cholesterol acyltransferase did not interfere with or affect the rate of appearance of radioactivity in the serum from the erythrocyte.

10. The pattern of distribution of radioactivity among the serum phospholipid classes after exchange with erythrocyte membrane preparations was similar to that of intact cells. Exchange of [^{3}H] palmityl-, [^{14}C] linoleyl- and [^{32}P] lecithin took place at markedly increased rates. The relative exchange rates of the former two lecithins compared to [^{32}P] lecithin were greater than unity suggesting that both these lecithins became entirely available for exchange.

11. The fractional rates of exchange of lecithins labelled by esterification *in vitro* of [^{3}H] palmitate and [^{14}C] linoleate and by exchange of [^{32}P] lecithin *in vivo*, increased with the *in vivo* age of the red cell, without change in the relative exchange rates of the acyl-labelled lecithins compared to that of [^{32}P] lecithin. The distributions of labelled phospholipids in the serum after exchange with either young or old red cells were similar. The pattern of labelling of [^{3}H] palmitate and ^{32}P among cellular phospholipids varied with red cell age. The proportion of [^{3}H] palmitate incorporated into phosphatidylethanolamine increased with age but that of [^{32}P] phospholipid decreased. This is in contrast to the similarity of distribution of [^{14}C] linoleate among the phospholipids of young and old cells.

12. In vivo, lecithin was found to exchange at rates lower than that observed in vitro and the relative exchange rates of [^{3}H] palmityl- and [^{14}C] linoleyl-lecithin were 1.03 ± .03 and 0.90 ± .01 respectively. A similarity between the in vitro and in vivo findings was that in both cases [^{14}C] linoleyl-lecithin exchanged at rates lower than that of [^{32}P] lecithin.

13. The data presented in this study suggest that all erythrocyte lecithin which is available for exchange with the serum, is located on the exterior surface of the erythrocyte membrane. Esterification of certain fatty acids with lysolecithins takes place in part at the interior surface of the red cell membrane, inaccessible to serum phospholipids.

6. APPENDIX

DESCRIPTION OF A TYPICAL IN VITRO EXCHANGE EXPERIMENT

The data to be shown in this appendix represent those obtained in a typical "basic *in vitro* exchange experiment". Other experiments in which for example, exchange in membrane preparations, exchange in both the inward and outward directions, or exchange in young and old red cells, were examined, the "typical basic *in vitro* experiment" served as a control. Rabbit erythrocytes were labelled with [^{32}P] phospholipids by exchange *in vivo* after intravenous injection of 10 mCi of $Na_3{}^{32}PO_4$. 3-4 days after injection the cells were collected using 0.25 M citrate as the anticoagulant and washed with ice-cold saline three times removing the buffy coat and the top 10% of the red cell column each time. One volume of packed washed ^{32}P-labelled red cells was incubated in 5 volumes of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 8 mM glucose for 2 hrs. at 37°C with a gas phase of 95% O_2, 5% CO_2. 30 µCi [9,10-^{3}H] palmitate and 10 µCi [1-^{14}C] linolenate or [1-^{14}C] linoleate bound to defatted BSA (3% solution) (244) were added per ml of incubated packed cells. After pre-incubation the cells were washed 3 times with 2% defatted BSA in Krebs-Ringer bicarbonate to remove unincorporated free fatty acids. After washing, aliquots were taken for individual exchange incubation samples, usually two for "zero time" controls and two for 4 hour samples. 1 ml packed cells were incubated with 4 ml serum at 37°C for 4 hrs. Zero time controls were performed by mixing the serum and cells, and then separating immediately. After separation and washing, the lipids of each serum and erythrocyte fraction were extracted by the Folch technique (253) in 20 volumes of chloroform-methanol 2:1, followed by concentration and separation into neutral lipids and phospholipids by silicic acid chromatography [Hirsch and Ahrens (254)]. The phospholipid fractions, each of which had been eluted with 200 ml methanol, were concentrated to dryness under a steam of nitrogen, and spotted on thin

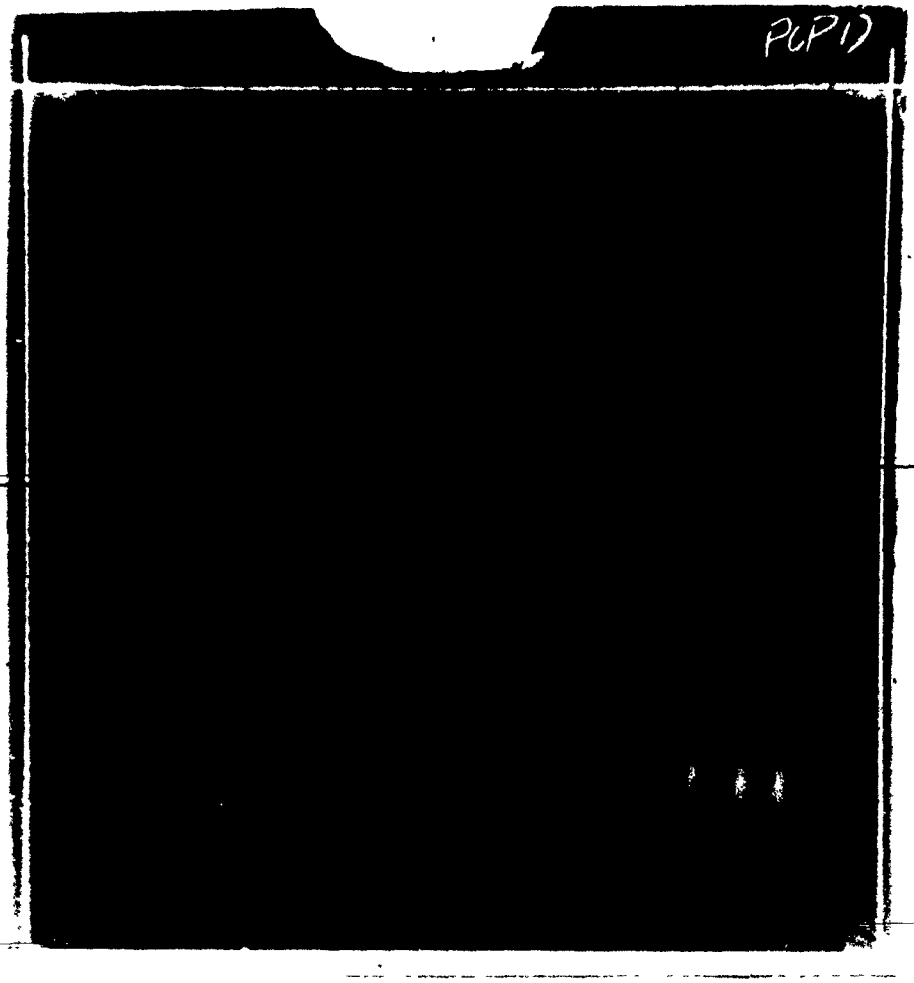


Figure 7: Thin Layer Chromatography of Serum Phospholipids on Binder-Free Silica Gel: Solvent System; Chloroform-Methanol-Acetic Acid-Water, 100:60:16:8.

1. Phosphatidylethanolamine 2. Phosphatidylserine and phosphatidylinositol
3. Lecithin 4. Sphingomyelin 5. Lysolecithin

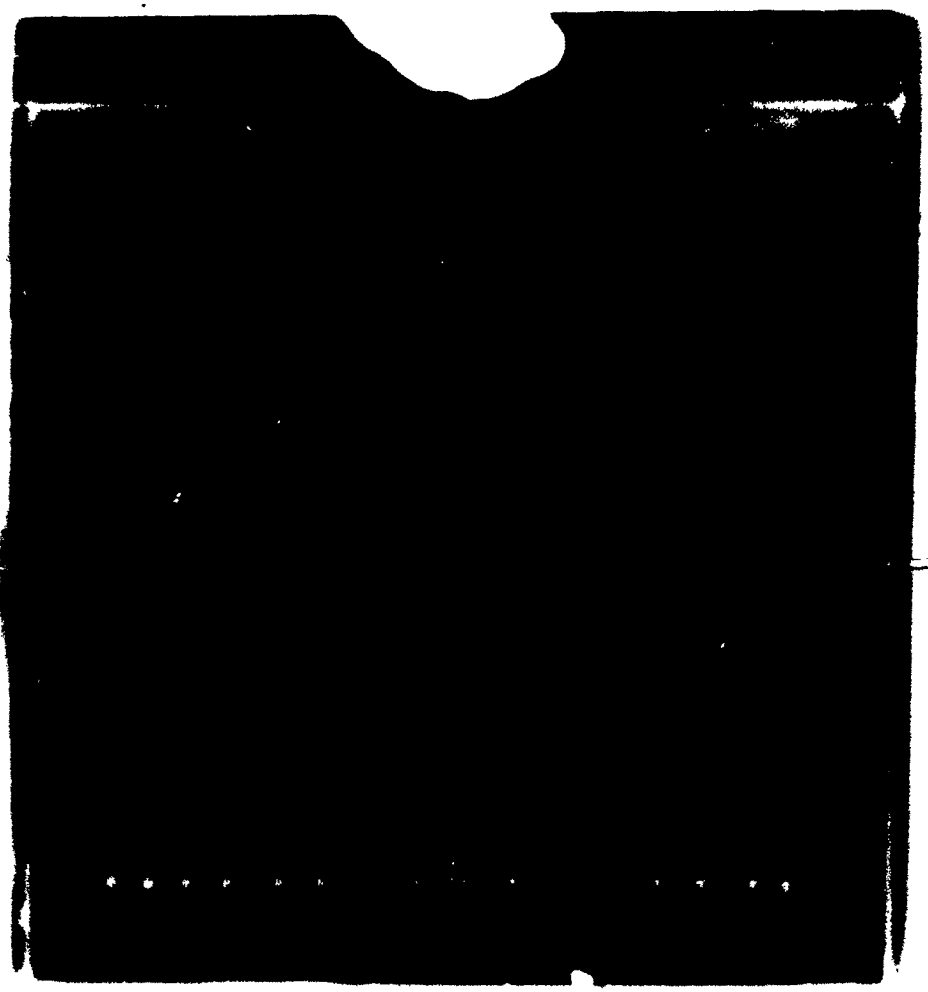


Figure 8: Thin Layer Chromatography of Erythrocyte Phospholipids: Chromatographic Conditions Are The Same as Outlined in Figure 7. 1. Phosphatidylethanolamine 2. Phosphatidylserine and phosphatidylinositol 3. Lecithin 4. Sphingomyelin 5. Lysolecithin

layer chromatographic plates, one plate per lipid extract, for isolation of the individual phospholipid classes. Typical samples of serum and erythrocyte phospholipids separated by TLC are shown in Figures 7 and 8 respectively. It was found that application of any more than one extract per plate overloaded it. This was unfortunate in view of the fact the average exchange experiment consequently required about 3-6 weeks to complete. After marking, the plates were deiodinated by standing overnight, scraped and the lipids eluted from each band. When appropriate, a small aliquot was taken for phosphorus determination and the remainder of extract was counted. In experiments in which lecithin was to be further fractionated into molecular species according to degree of unsaturation, the lecithin samples were concentrated and spotted on silver nitrate-impregnated plates, one sample per plate. Again, the possibility of overloading did not allow any more than one sample per plate. A typical separation is shown in the photograph of Figure 9. For illustrative purposes; egg lecithin and dilinoleyl lecithin were also spotted at the extreme left of the plate. After the lipids from the TLC plates were recovered from the appropriate plates they were counted in the Packard Tri-Carb liquid scintillation counter. Spillage from the ^{32}P channel into the ^{14}C and ^{3}H channels was about 15-20% and 0.5-1.5% respectively. Spillage from ^{14}C channel into ^{3}H channel was about 5-10%. The spillage for each individual experiment was precisely determined each time. At any rate the level of radioactivity in each channel was usually in the order, $^{3}H > ^{14}C > ^{32}P$, so that the actual correction of counts on the average was usually about 5% for both ^{3}H and ^{14}C. The windows were set so that there was virtually no spillage from ^{3}H to ^{14}C to ^{32}P. Typical data from a standard "basic in vitro exchange experiment" are shown in Tables XVIII and XIX.

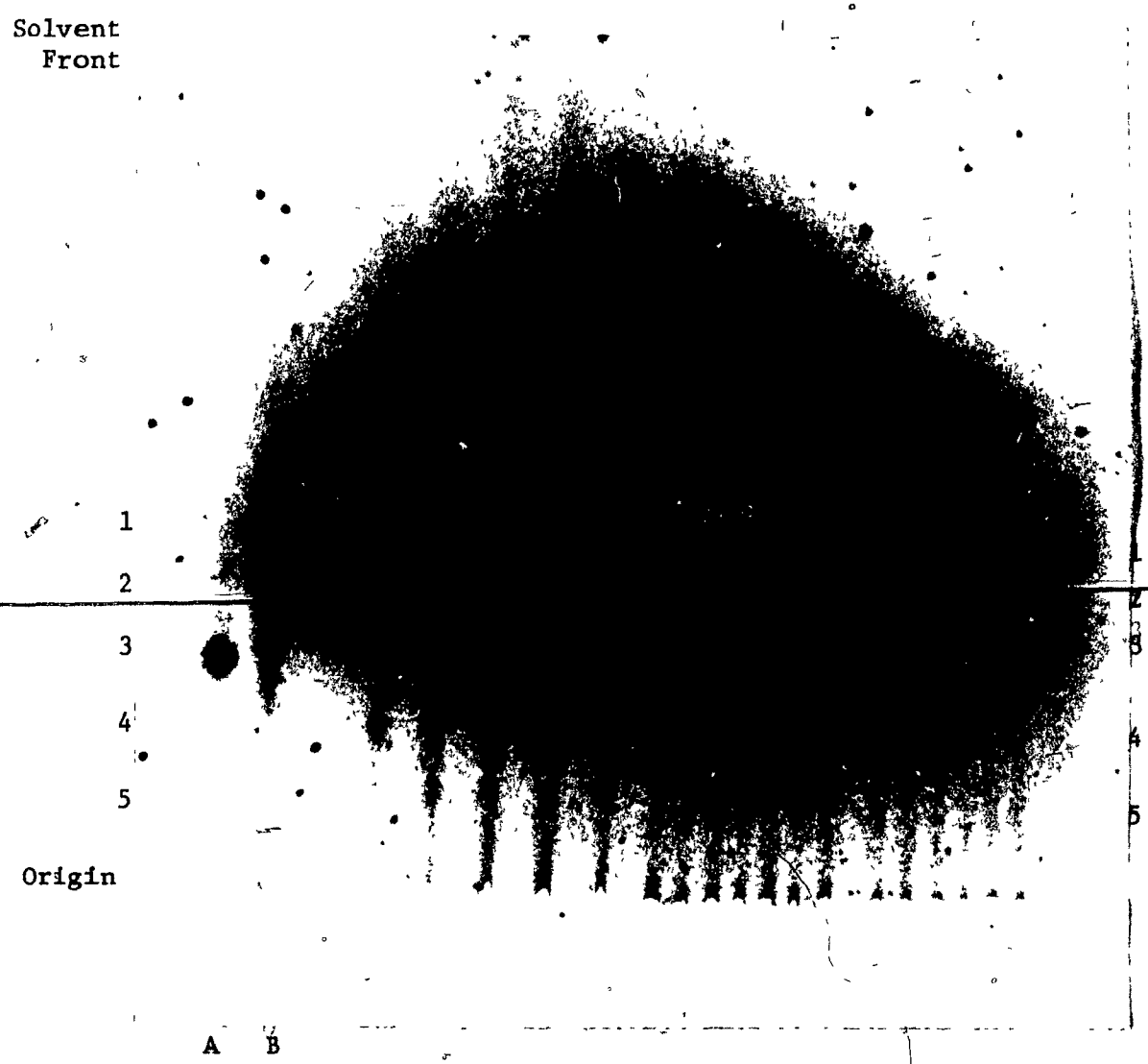


Figure 9: Argentation Thin Layer Chromatography of a Sample of Lecithin on Silver Nitrate-Impregnated Thin Layer Plates (15g $AgNO_3$/50g Binder-Free Silica Gel): Solvent System; Chloroform-Methanol-Water 60:30:8. This particular sample was Erythrocyte Lecithin. Synthetic Dilinoleyl Lecithin and Egg Lecithin Were Run at The Extreme Left of the plate for illustrative purposes at Positions A and B respectively.

1. Saturated and Monoenoic 2. Dienoic 3. Tetraenoic 4. Hexenoic
5. Polyenoic Lecithins

TABLE XVIII

Radioactivity* in Serum Phospholipids After Control and 4 hr. Incubation with Erythrocytes Labelled with ^{32}P, [^{3}H] Palmitate and [^{14}C] Linoleate

Sample Description	Phospholipid	Radioactivity of Lecithin (Cpm) Precursor [^{3}H] Palmitate	[^{14}C] Linoleate	^{32}P
Zero Time Control No. 1	Lysolecithin (LPC)	70	20	10
	Sphingomyelin (S)	30	10	10
	Lecithin (PC)	1,860	1,000	80
	Phosphatidylserine + (PS)			
	Phosphatidylinositol (PI)	140	40	0
	Phosphatidylethanolamine (PE)	630	250	10
Zero Time Control No. 2	LPC	100	10	20
	S	60	20	10
	PC	2,300	1,100	100
	PS + PI	100	30	0
	PE	680	250	10
4 hr. Sample No. 1	LPC	760	210	150
	S	270	70	120
	PC	19,800	9,250	1,700
	PS + PI	420	170	20
	Pe	1,800	840	50

Continued

TABLE XVIII cont'd

	LPC	680	180	140
4 hr.	S	240	110	130
Sample	PC	12,900	6,900	1,230
No. 2	PS + PI	900	290	30
	PE	3,300	1,100	40

* corrected for spillage, ^{32}P decay, and when necessary, quenching, (also for reduced efficiency due to reduction of window width).

TABLE XIX

Radioactivity in Erythrocyte Phospholipids After Control and 4 hr. Incubation with Erythrocytes Labelled with ^{32}P, [^{3}H] Palmitate, and [^{14}C] Linoleate

		Radioactivity of Lecithins (Cpm)		
		Precrusor		
Sample Description	Phospholipid	[^{3}H] Palmitate	[^{14}C] Linoleate	^{32}P
Zero	LPC	2,400	2,000	570
Time	S	8,400	4,500	25
Control	PC	273,000	230,000	25,900
No. 1	PS + PI	12,100	9,000	1,000
	PE	195,000	78,800	6,000
Zero	LPC	2,700	2,200	620
Time	S	10,700	7,000	2,500
Control	PC	262,000	215,000	24,300
No. 2	PS + PI	16,500	11,200	1,100
	PE	181,000	74,600	5,500
	LPC	1,400	2,800	410
4 hr.	S	6,400	4,200	2,400
Sample	PC	257,600	225,000	26,100
No. 1	PS + PI	18,600	14,700	1,400
	PE	210,000	85,600	6,700
	LPC	1,200	1,400	500
4 hr.	S	4,800	2,900	2,200
Sample	PC	225,000	219,000	25,700
No. 2	PS + PI	18,500	12,300	1,200
	PE	207,000	82,600	6,500

CLAIMS TO ORIGINALITY

The following are considered to be contributions of original knowledge.

1. The rate of phospholipid exchange between the erythrocyte and serum lipoproteins depends on the phospholipid concentration of the medium but not on any specific class of serum lipoprotein.

2. Lecithin is the principal phospholipid exchanged between the rabbit erythrocyte and serum. The rate of exchange of esterified erythrocyte lecithins varies with the fatty acid precursor, but no esterified lecithin studied exchanged at a rate greater than that of ^{32}P-labelled lecithin, which was originally incorporated by exchange.

3. [^{14}C] linoleate, [^{14}C] linolenate, [^{3}H] palmitate, and [^{32}P] lecithin are each incorporated by the erythrocyte into the various molecular species of lecithin, being distributed among them in patterns specific for each precursor. Subsequent exchange of these lecithins into serum also results in characteristic distributions among the various molecular species of serum lecithins.

4. Although the fractional lecithin exchange rates of erythrocyte membrane preparations are three times that of intact cells, the difference in fractional exchange rates between [^{3}H] palmityl- or [^{14}C] linoleyl-lecithin and [^{32}P] lecithin is reversed in the membranes, suggesting that a portion of the total lecithin pool becomes available for exchange only upon disruption of the erythrocyte and may be located in a relatively inaccessible portion of the membrane such as the inner surface.

5. The fractional exchange rates of acyl-labelled and ^{32}P-labelled lecithins increase with the in vivo age of the red cell without alteration in the exchange rates of these lecithins relative to each other. The distribution of

labelled phospholipids in the serum is similar after exchange with either young and old red cells despite the variation with age in the pattern of labelling of certain precursors among the erythrocyte phospholipids. It is suggested that alterations in the erythrocyte membrane which may occur as the red cell ages do not affect the intactness of the cell.

6. In vivo, erythrocyte lecithin exchanges at rates lower than that observed in vitro and [^{14}C] linoleyl lecithin exchanges at a somewhat lower rate than that of [^{3}H] palmityl lecithin and [^{32}P] lecithin, which are similar in their rates.

7. In addition to the above original contributions, some properties of phospholipid exchange which have been observed for certain mammalian species have now been extended to the rabbit. Exchange of radioactive phospholipid between the rabbit erythrocyte and serum in vitro is linear with time for at least 6 hrs. About 55% of the total cellular lecithin of the rabbit erythrocyte is available for exchange. Serum phospholipase and lecithin cholesterol acyltransferase activity do not produce artefacts in the study of lecithin exchange. After exchange in vitro, at least 80% of the serum radioactivity of [^{3}H] palmitate-, [^{14}C] linoleate-, (^{14}C] linolenate-, and ^{32}P-labelled phospholipids is present in lecithin.

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