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METABOLISM OF RED-CELL LIPIDS

III. PATHWAYS FOR PHOSPHOLIPID RENEWAL

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SUMMARY

1. Experiments on the conversion of various phospholipid precursors by red-cell ghosts indicated that monoacyl phosphoglycerides are the major acyl acceptors involved in the fatty acid incorporation into lipids of mature mammalian erythrocytes.

2. Lysates of red cells were incapable of catalyzing the hydrolysis of lecithin into the monoacyl derivative. Lysolecithin derived from the plasma by an exchange reaction was found to be acylated to some extent by intact erythrocytes so as to form lecithin.

3. The renewal of phospholipids from erythrocytes is considered to involve at least two processes: (a) exchange of phospholipid molecules between serum and cells, (b) a positionally specific incorporation of saturated and unsaturated fatty acids with the participation of monoacyl phosphoglycerides originating from the serum.

4. The extent of these processes was found to be different for distinct classes of phospholipids, while even within one phospholipid class not all molecular species participated in the exchange reaction. The magnitude of phospholipid renewal appears to differ between erythrocytes of various mammalian species.

INTRODUCTION

Recent studies from several laboratories have established that red blood cells or their post-hemolytic residues are capable of incorporating fatty acids into their phosphoglycerides[1-6]. Furthermore, red-cell ghosts were found to catalyze a conversion of monoacyl phosphoglycerides (*e.g.* lysolecithin) into the diacyl analogs[2,5,7,8]. Experiments with doubly-labeled lysolecithin unequivocally demonstrated that the incorporation of fatty acids into phospholipids of red cells which requires ATP and CoA is linked to an acylation of lyso derivatives[8]. It remained to be established, however, whether this reaction is exclusively responsible for the introduction of fresh fatty acid constituents into these red-cell lipids.

Apart from a partial renewal of phospholipid molecules by synthetic reactions, the erythrocyte membrane appears to be able to exchange complete phospholipid units with its environment[9-13]. A possible relationship between both types of processes has now been investigated in mature non-nucleated red cells.

MATERIALS AND METHODS

[^{32}P]Phosphorylcholine was obtained by degradation of [^{32}P]lecithin[8] with phospholipase C (EC 3.1.4.3) from *Bacillus cereus*[14]. The substrate was emulsified by ultrasonic irradiation, and, after complete breakdown (which required a 2-h incubation period), the glycerides were extracted into ether. The water layer was brought to dryness and the residue treated with methanol. The phosphorylcholine thus obtained revealed upon paperchromatography a radiochemical purity of over 95%.

[^{14}C]Diglycerides were prepared by enzymic degradation of red-cell lecithin containing ^{14}C-labeled fatty acids. After a complete hydrolysis with phospholipase C, the lipid-soluble material was extracted with ether. In order to avoid unwanted migrations of the fatty acid residues, the diglycerides were not purified by means of silica chromatography. Thin-layer chromatograms[6] showed one radioactive spot coinciding with that of 1,2-diglycerides.

Glycero-3-[^{32}P]phosphorylcholine was obtained after weak alkaline hydrolysis of lecithin according to the method of DAWSON[15]. The radiochemical purity of the compound was about 95%.

The preparation of labeled lecithin, lysolecithin and lysophosphatidyl ethanolamine has been described previously[8].

Erythrocytes and plasma containing [^{32}P]phospholipids were obtained by intravenous injection into rats of two doses of 300 μC Na_2HPO_4 with an interval of 7 h. The blood was withdrawn after 24–36 h, and the blood cells were separated by centrifugation. The cells were washed 3 times with isotonic saline and the erythrocytes were freed from leucocytes and reticulocytes by ultracentrifugation[16]. Rabbits were injected with 7–10 mC Na_2HPO_4, and the blood collected after 24 h was worked up in a similar way.

The incubation and analytical procedures were similar to those utilized in our previous studies[6,8].

RESULTS

When compared with the uptake of fatty acids the incorporation of phosphate into the phospholipids of non-nucleated red cells *in vitro* appears to be of a low order (Fig. 1). Some differences were found in the magnitude of phosphate incorporation into the red-cell lipids of different mammalian species; also the nature of the phospholipid labeled with radioactive phosphate appeared to differ (Table I). After paperchromatographic separation of the phospholipids from human and rabbit erythrocytes the radioactivity was found to coincide with a spot having the mobility of phosphatidic acid. Alkaline hydrolysis indeed gave rise to the formation of [^{32}P]glycerophosphate. As regards the other animal species studied the radioactivity incorporated into the red-cell phospholipids was not usually sufficient to allow any final conclusion, though in a number of experiments lecithin was found to be labeled to a small extent. In general our experiences are in agreement with the studies of RADERECHT *et al.*[17], who concluded that the incorporation of phosphate into phospholipids decreases significantly during maturation of red cells. As regards the pathways of phosphatidic acid formation in human erythrocyte ghosts, reference can be made to the studies of HOKIN AND HOKIN[18] who observed that phosphorylation of 1,2-diglycerides is about

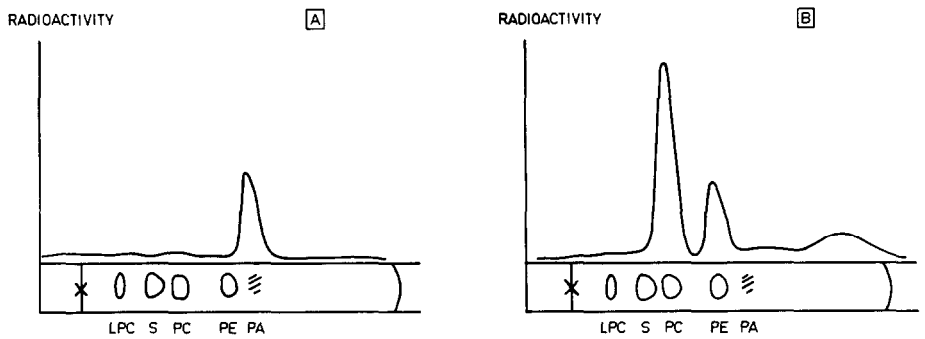


Fig. 1. Radiochromatograms demonstrating the incorporation of [^{32}P]phosphate and ^{14}C-labeled fatty acid into phospholipids of mature rabbit erythrocytes. Equal amounts of erythrocytes and Ringer solution (without inorganic phosphate) were incubated for 5 h at 37° with 10 μC of $Na_2H^{32}PO_4$ (nearly carrier free) and 1 μC of [1-^{14}C]linoleic acid (containing 13.7 μg fatty acid) respectively. Abbrevations: LPC, lysolecithin; S, sphingomyelin; PC, lecithin (or phosphatidylcholine); PE, phosphatidyl ethanolamine; PA, phosphatidic acid.

2500 times more active than synthesis by an acylation of glycero-3-phosphate.

The view that a synthesis *de novo* of quantitatively major phospholipids is of a low order in the mature non-nucleated red cell was supported by the results com-

TABLE I

INCORPORATION OF $^{32}PO_4$ *in vitro* INTO PHOSPHOLIPIDS OF ERYTHROCYTES OF MAN AND VARIOUS ANIMALS

Incubation mixtures contained 5 ml of erythrocytes, 5 ml of Ringer solution (without inorganic phosphate), 10 mg of glucose, 100 μC of [^{32}P]orthophosphate, 5000 IE of penicillin and streptomycin, and were incubated for 5 h at 37° under atmospheric conditions. (The values are the means of at least 4 experiments.)

	Activity in counts/min/μg phosphate	*Major radioactive phospholipid formed*
Human	13	Phosphatidid acid (>90%)
Rabbit	25	Phosphatidic acid (>90%)
Rat	<1	Lecithin
Pig	<0.2	Lecithin
Ox	<0.2	Lecithin
Sheep	<0.2	Lecithin

TABLE II

COMPARISON OF INCORPORATION OF PHOSPHOLIPID PRECURSORS INTO LECITHIN OF ERYTHROCYTE GHOSTS

The incubation medium consisted of 0.5 ml of intact or ultrasonically hemolyzed rabbit erythrocytes in 0.5 ml Ringer solution containing 0.1 μmole of CoA, 5 μmoles of ATP, 100 μg of linoleic acid, 100 μg of palmitic acid and trace amounts of the labeled substrates. In Expts. 4 and 5, 0.3 μmole of cytidine-diphosphate choline was added. The mixture was incubated at 37° for 5 h under continuous agitation.

Substrate	*Incorporation (%)*
1 $Na_2H^{32}PO_4$	<0.001
2 Glycero-3-[^{32}P]phosphate	<0.01
3 [^{32}P]Phosphorylcholine	<0.3
4 Glycero-3-[^{32}P]phosphorylcholine	<0.3
5 1-Acyl-2-[1-^{14}C]linoleoyl-glycerol	<1
6 1-Acyl-2-[1-^{14}C]oleoyl-glycerol	<2
7 1-Acyl-glycero-3-[^{32}P]phosphorylcholine	30–40

piled in Table II indicating a limited conversion of glycero-3-phosphate, phosphorylcholine and diglycerides to phospholipid by red-cell ghosts. In contrast to the poor incorporation of these phospholipid precursors the mature mammalian erythrocyte appears rather actively to metabolize lysolecithin and its analogs, thereby indicating that the fatty acid incorporation into red-cell phospholipids is attributable mainly to a transacylation of lysophosphoglycerides. This conclusion is supported by the observation that the uptake of fatty acids by erythrocytes was enhanced by the addition of lysolecithin whereas glycero-3-phosphate failed to give any stimulation[4,6]. That this increase in fatty acid incorporation represents a specific stimulation is demonstrated by the effects produced by different classes of monoacyl phosphoglycerides. The addition of lysolecithin was found to stimulate the incorporation of linoleic acid

TABLE III

EFFECT OF LYSOPHOSPHOGLYCERIDES ON THE INCORPORATION OF [^{14}C]LINOLEIC ACID INTO ERYTHROCYTE PHOSPHOLIPIDS

Incubation mixtures containing 0.5 ml of hemolyzed erythrocytes in 0.5 ml of Ringer solution and 0.1 ml of a solution containing 50 μmoles of ATP/ml, 1 μmole of CoA/ml, 4 μC of 1-[^{14}C]linoleic acid, lysolecithin (1 mg) and lysophosphatidyl ethanolamine (0.3 mg) were added. The values represent averages of 4 experiments.

	Distribution of radioactivity (%)	
	Lecithin	*Phosphatidyl ethanolamine*
Rabbit erythrocytes		
No addition	85	15
Lysolecithin	98	2
Ox erythrocytes		
No addition	73	28
Lysophosphatidyl ethanolamine	50	50

into lecithin of rabbit erythrocyte ghosts, whereas in ox erythrocyte ghosts lysophosphatidyl ethanolamine caused an increase in linoleic acid uptake into phosphatidyl ethanolamine (Table III). Similar results have been reported recently by ROBERTSON AND LANDS[5] for the incorporation of fatty acids into red-cell ghosts of man and sheep.

The various observations strongly support the concept that lysophosphoglycerides act as major acyl acceptors in mature erythrocytes of different mammalian species. The question arises whether these intermediates are formed *in situ*, thus giving rise to the action of a phosphoglyceride cycle in erythrocytes similar to that observed in rat liver[19]. However, we were not able to detect any appreciable phospholipase A (EC 3.1.1.4) activity in red-cell ghosts, this being in agreement with the observations of OLIVEIRA AND VAUGHAN[4] and ROBERTSON AND LANDS[5]. Both the formation of [^{32}P]lysolecithin and the liberation of ^{14}C-labeled fatty acids from correspondingly labeled lecithins in the presence of erythrocyte ghosts did not exceed the non-enzymic breakdown observed in the control experiments (Table IV). On the other hand a small but definite degradation of lecithin was found to be caused by plasma. A low phospholipase-A-like activity in human serum has been described by ZIEVE AND VOGEL[20,21] and by ETIENNE *et al.*[22]. Cholesterol esterification in plasma of man and rat has been found by GLOMSET *et al.*[23-25] to involve a transfer of fatty acid from the 2-ester position of lecithin thus giving rise to the formation of lysolecithin. A relatively high content of lysolecithin in lipid extracts of plasma has been

TABLE IV

COMPARISON OF PHOSPHOLIPASE-A-LIKE ACTIVITY OF RABBIT ERYTHROCYTES AND PLASMA

0.5 ml of hemolyzed rabbit erythrocytes was incubated for 5 h in 0.5 ml of Ringer solution, mixed with labeled substrate. Replacement of Ringer solution by acetate buffer (pH 6) and addition of deoxycholate, EDTA, acid phospholipid (cardiolipin) and unlabeled lysolecithin did not alter the results obtained with erythrocytes.

	[^{32}P]*Lysolecithin formed from* [^{32}P]*lecithin* (%)	[^{14}C]*Fatty acids liberated from* [*acyl-*^{14}C]*lecithin* (%)
Hemolyzed rabbit erythrocytes	1–3	3–4
Plasma	10	<5
Control	1–2	2–4

repeatedly reported, and NEWMAN *et al.*[26] provided strong evidence for the physiological occurrence of lysolecithin in rat plasma. MISRA[27] has detected small quantities of lysophosphatidyl ethanolamine in human serum.

Since erythrocyte ghosts appear to be devoid of phospholipase A activity, and since lysophosphoglycerides such as lysolecithin are abundant in the red-cell environment, it seems plausible that these acyl acceptors are transferred to the red cell by means of exchange reactions known to occur between blood cells and serum lipoproteins[9–13]. For this reason the phospholipids of erythrocytes and serum were labeled by intravenous injection of [^{32}P]phosphate into rats. By contrast to an incubation *in vitro* of erythrocytes with radioactive phosphate the administration of this marker into living animals manifested after a few hours a significant labeling of the red-cell phospholipids. Paper chromatograms showed that in both erythrocytes and plasma, lecithin and lysolecithin were the major radioactive lipids. After incubation of unlabeled rat erythrocytes with labeled plasma a considerable part of the radioactive phospholipids was recovered from the erythrocytes (Fig. 2). Conversely, incubation of inactive plasma with erythrocytes labeled *in vivo* or *in vitro* revealed a transfer of the radioactive phospholipids into the medium. In these experiments both lecithin and lysolecithin were found to participate in the interchange between red cells and plasma but the exchange of lysolecithin was found to prevail. These observations are in fair agreement with the results of POLONOVSKI AND PAYSANT[13] who reported a rapid exchange of lysolecithins between blood cells and plasma of the rat. It is difficult to evaluate exactly the quantity of phospholipids adsorbed to the surface of the red cell, though the amount was minimized as much as possible by washing procedures.

Similar experiments carried out with erythrocytes and plasma from other animals showed that significant quantitative differences may exist in the exchange process between different mammalian species. The erythrocytes and plasma obtained from rabbits 24 h after injection of radioactive phosphate showed some differences in distribution of radioactivity among the various classes of phospholipids though lecithin in both tissues appeared to contain more than 50% of the radioactivity. Experiments carried out 48 h after the injection of [^{32}P]phosphate showed as much as 70% of the radioactivity of the phospholipids from erythrocytes and plasma to be located in lecithin. Furthermore, lysolecithin was found to be labeled, and some radioactivity was detected also in phosphatidyl ethanolamine and sphingomyelin. Incubation of erythrocytes together with plasma again revealed an exchange of phospholipids, particularly of lecithin, though lysolecithin was also found to participate.

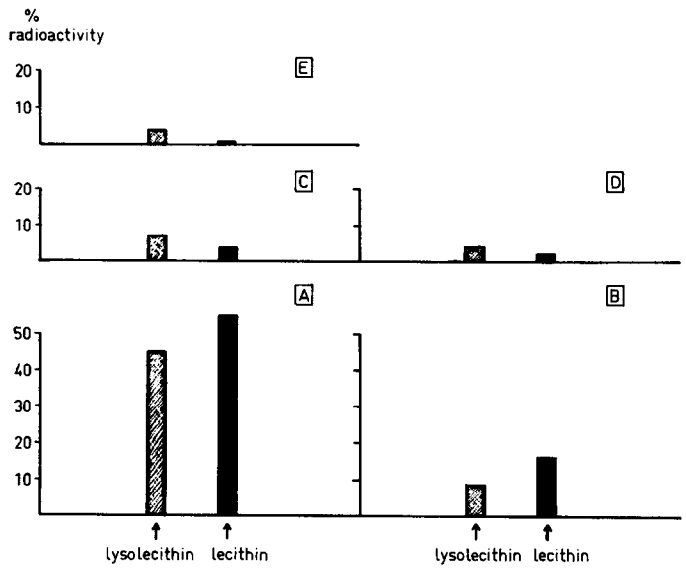


Fig. 2. Schematic representation of exchange of [^{32}P]lecithin and [^{32}P]lysolecithin between plasma and red cells of rat. The radioactivity of equal volumes of erythrocytes and plasma is compared. Incubation procedures: erythrocytes and plasma labeled *in vivo* were prepared as described under METHODS. Equal volumes of washed erythrocytes and plasma were incubated for 5 h at 37°. After separation of cells and plasma the lipids were extracted, and the radioactivity of lecithin and lysolecithin was determined on paper chromatograms. A, plasma labeled *in vivo*; B, erythrocytes labeled *in vivo*; C, unlabeled erythrocytes incubated *in vitro* with labeled plasma of A; D, unlabeled plasma incubated *in vitro* with labeled erythrocytes of B; E, unlabeled plasma incubated *in vitro* with labeled erythrocytes of C. (The mean is given of 5 separate experiments.)

in this process. Incubation of sheep erythrocytes in rabbit plasma containing labeled phospholipids gave only a very slight transfer of phospholipids into the red cells, lysolecithin being the major component. Though not conclusive, the results recorded above indicate that erythrocytes may take up from the serum the lysophosphoglycerides required for transacylation. Actually, after injection of radioactive phosphate into rats and rabbits, the ratio of activity between lecithin and lysolecithin in erythrocytes was found to increase with time. The assumption that lysolecithin after its translocation from the serum to the red cell is acylated so as to form lecithin can be verified by investigations *in vitro*. When experiments were carried out with plasma having all phospholipids labeled, this approach was hampered to some extent because of an interference of the exchange of diacylphosphoglyceride molecules (particularly lecithins) between plasma and cells. For this reason erythrocytes of rat and rabbit were incubated in plasma to which a trace amount of [^{32}P]lysolecithin had previously been added. According to expectation, the erythrocytes became labeled not only in the lysolecithin fraction, but in addition the paperchromatograms showed a small but definite amount of [^{32}P]lecithin to be present (Fig. 3). Control experiments carried out under identical conditions with plasma alone did not reveal any conversion of lysolecithin into lecithin. Hence, it may be concluded that erythrocytes of the mammals studied are capable of utilizing lysolecithin derived from plasma for synthesis of lecithin. Examination of the water-soluble fraction showed that part of the lysolecithin substrate was degraded into glycerylphosphorylcholine. The formation of this

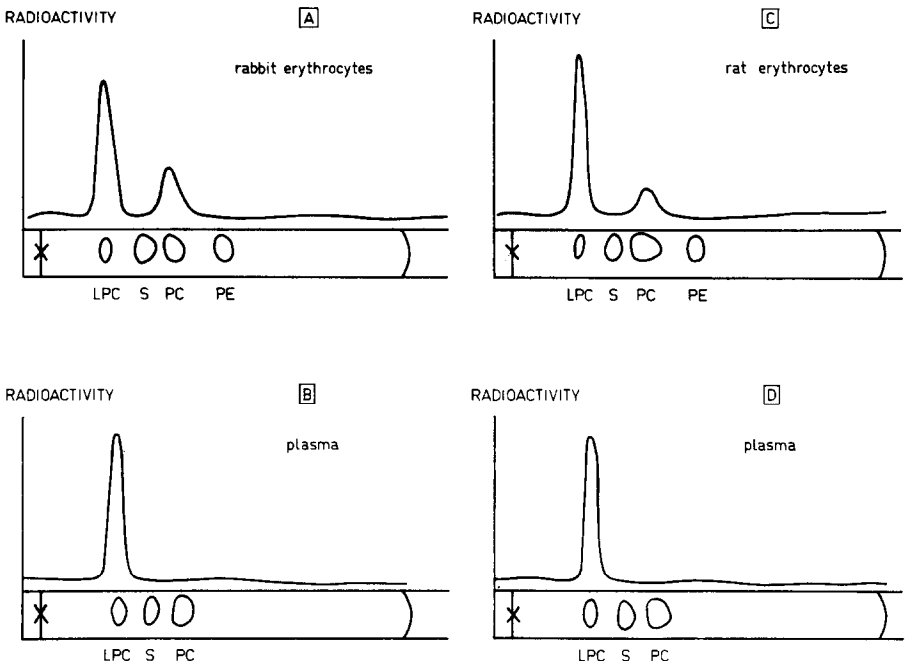


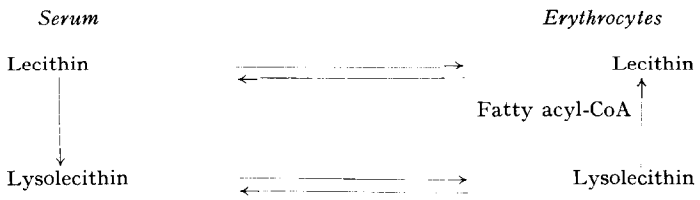
Fig. 3. Distribution of ^{32}P activity on radiochromatograms of phospholipids from rabbit and rat erythrocytes after incubation of intact cells in plasma with a trace amount of 1-acyl-glycero-3-[^{32}P]phosphorylcholine. Experiments B and D were control experiments carried out with plasma alone.

phosphodiester is in agreement with previous observations on the lysophospholipase activity (EC 3.1.1.5) of blood cells[7,8] and serum[22].

DISCUSSION

The renewal of fatty acid constituents of red-cell phospholipids *in vitro* appears to involve at least two processes: (a) an enzymically controlled incorporation of fatty acyl-CoA derivatives into phosphoglycerides; (b) exchange of phospholipids between erythrocytes and serum. As regards the first phenomenon the present study supports the view that (trans)-acylation of monoacyl phosphoglycerides is responsible mainly for the uptake of fatty acids into the phospholipids of intact erythrocytes and red-cell ghosts. However, a complete enzyme system catalyzing a mono-diacyl phosphoglyceride cycle, as found in rat liver[19], appears not to be acting in the red-cell membrane. Not only the fatty acid constituents but also the monoacyl phosphoglycerides appear to be provided by the serum. In this respect the second process involving a transfer of phospholipids from the serum lipoproteins to the red-cell membrane and *vica versa* is of particular interest. This exchange of phospholipid molecules, which itself represents an important pathway for the replacement of membraneous constituents, appears to supplement the enzymically regulated renewal of fatty acid residues by donating the acyl acceptors and withdrawing surplus phospholipid molecules.

The information available at present concerning the dynamic events of lecithin in the red cell membrane has been summarized tentatively in Scheme 1, omitting the degradation of lysolecithin into glycerophosphorylcholine and the conversion:



Scheme 1. Pathways for the renewal of lecithin in the red-cell membrane. Not all lecithin species appear to be involved, and significant quantitative differences exist in these processes between erythrocytes from different mammalian species.

2 lysolecithin → lecithin + glycerylphosphorylcholine[8]. This concept combining the exchange of phospholipid molecules and metabolic conversion of lyso derivatives by the red cell accounts fairly well for the various observations made in the present series of investigations and the results of other studies on single reactions referred to above.

As regards the chemical structure of the lysolecithin delivered to the red-cell membrane for acylation, the positionally specific incorporation of saturated and unsaturated fatty acids suggests that both 1-acyl-glycero-3-phosphorylcholine and 2-acyl-glycero-3-phosphorylcholine are involved. Recent studies in this laboratory showed that these structurally isomeric lyso compounds occur in rat liver[19], and it will be of interest to extend these investigations to lysolecithin from blood. The lysolecithins may be formed by enzymic breakdown of lecithin in serum, but it is not unlikely that these compounds originate in part from the liver. In principle the given sequence of reactions may apply to other types of phosphoglycerides as well, although the magnitude of various processes may be different. It is worth noting that the incorporation of fatty acids into phosphatidyl ethanolamine in general is much less than that into lecithin of red cells, while the content of lysolecithin in serum is known to dominate that of the ethanolamine analog. No incorporation has been observed of fatty acids into red-cell sphingomyelin. This may be due to the absence of either a specific trans-acylase or the deacylated precursor. DAWSON[28] concluded that sphingosylphosphorylcholine is not present in animal tissues. Therefore the renewal of sphingomyelin in the red-cell membrane as opposed to that of phosphoglycerides may depend on the exchange process only.

Furthermore it needs emphasis that within one class of phosphoglycerides not all molecular species have to participate to the same extent in the various reactions given in Scheme 1. As regards the exchange of lecithin between the cell membrane and serum lipoproteins, some limitations became apparent when comparing the fatty acid composition of this phospholipid from both origins. Confirming the observations and conclusions of ROWE[12] a distinction has been found in the fatty acid pattern of lecithin from red cell and serum both in man and animal[29]. In addition, current dietary investigations showed that alterations in the nature and amount of ingested fats induced quantitatively different effects in the fatty acid pattern of lecithin from red blood cells and serum. Hence, only a part of the lecithin molecules present in the lipoproteins of the serum and (or) the red-cell membrane appears to contribute to the interchange process.

In principle circulating erythrocytes may utilize both the exchange of complete phospholipid molecules and the enzymic trans-acylation of lysophosphoglycerides in order to renew these important constituents of the membrane. Though experiments *in vitro* suggest that in some animal species the former process may be the prevailing

one, detailed investigations are required to assess the relative quantitive importance of these co-operative mechanisms *in vivo*. In this respect attention has to be paid to quantitative differences in the composition and metabolism of phospholipids of erythrocytes between different mammalian species. As discussed already by VAN DEENEN AND DE GIER[30] the great variations in the lecithin content of erythrocytes as contrasted by the predominant occurrence of lecithin in the serum of all the animal species concerned appears to put some limitations on the quantitative importance of the exchange process for certain mammalian species. Actually our preliminary observations on the uptake of phospholipids by erythrocytes of rat, rabbit and sheep support the suggestion that the magnitude of phospholipid interchange between cells and serum differs significantly among different mammals. Furthermore, variations may exist in the activity of the enzymically governed fatty acid uptake between erythrocytes of different species. Although red-cell ghosts of a number of species were found to be about equally apt to convert lysolecithin into lecithin, the fatty acid incorporation into the phospholipids of intact erythrocytes appeared to differ significantly[6]. Further studies are required to elucidate the basis underlying these differences in composition and dynamic events of phospholipids from various mammalian erythrocytes. With a view to elucidating the intriguing process of the elimination of the circulating erythrocyte after a defined life span it may be of interest to investigate the various processes of membraneous phospholipids in erythrocytes of different ages.

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