Phospholipid Exchange Between Plasma and Erythrocytes in Man and the Dog

CLAUDE F. REED with the technical assistance of MARION MURPHY and GERALDINE ROBERTS

From the Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14620

ABSTRACT The turnover of the four major erythrocyte phospholipids has been studied with ^{32}P, both in vivo and in vitro, in man and the dog. Phosphatidyl serine and phosphatidyl ethanolamine appeared to be stable erythrocyte lipids in both species. Turnover of the phosphate moiety of lecithin and sphingomyelin in the circulating erythrocytes of these two species seems entirely due to an exchange of the whole molecule with the corresponding plasma compound. Exchangeable and nonexchangeable pools of these two cellular lipids were found. In man about 60% of erythrocyte lecithin is exchangeable. The 12 hr fractional turnover of this pool is approximately 13%. Only 30% of the sphingomyelin in human cells appeared exchangeable; this portion had a 12 hr fractional turnover of about 14%. Similar results were obtained in the dog except that in this species about 75% of the erythrocyte sphingomyelin was exchangeable. Inorganic ^{32}P was not incorporated into any of the four major phospholipids in either species. The present findings aid in estimating quantitatively the effect of plasma-erythrocyte lipid exchange on red blood cell phospholipids.

INTRODUCTION

There is now substantial agreement about the major classes and amounts of lipids present in human erythrocytes (2). While hemoglobin and

---

A preliminary report of this work has been presented previously (1).

Received for publication 8 August 1967 and in revised form 30 November 1967.

structural membrane protein are thought to be stable within the life-span of the circulating mature red blood cell (RBC) (3, 4), a number of reports have indicated that some exchange of erythrocyte lipid with corresponding plasma compounds occurs in various species.

Hevesey and Hahn, using the rabbit, (5) were the first to describe an exchange of phospholipids between erythrocytes and plasma. These workers (6) also suggested that the process was probably a slow one, and that all of the erythrocyte phospholipids were not involved equally. British workers have presented suggestive in vitro evidence that phospholipid exchange between cells and plasma occurs in human blood (7, 8). More recently such an exchange has been described in the rat by several groups (9–11). In all of the above reports the description of the process has been primarily qualitative, and actual rates of exchange for the individual phospholipids have not been determined.

The present study describes the dynamic state of the phosphate moiety of the four individual major phospholipids of circulating erythrocytes (sphingomyelin, lecithin, phosphatidyl ethanolamine, and phosphatidyl serine) in quantitative terms. The dog and man were studied by using ^{32}P as a label. The results of short-term in vitro studies of plasma and erythrocyte phospholipid exchange are compared to results obtained from more prolonged in vivo observations. It was found that erythrocyte lecithin and sphingomyelin exchange with the corresponding plasma compounds in both species, and it was possible to determine the rate of these processes. In addition, exchangeable and nonexchangeable fractions of

erythrocyte sphingomyelin and lecithin were found to exist. In contrast to these two lipids, phosphatidyl ethanolamine and phosphatidyl serine appeared to be stable membrane lipids.

METHODS

Normal human donors, patients with uncomplicated polycythemia rubra vera who had received therapeutic orthophosphate-^{32}P, and mongrel dogs were used as subjects. Blood was collected into Na$_2$EDTA (1.25 mg/ml of blood). The red blood cells were washed three times in 5 volumes of cold 0.17 M NaCl and the buffy coat was carefully removed each time. The white blood cell count was less than 500 per cubic mm and platelets were absent from the washed cells. The erythrocyte and plasma lipids were extracted, separated, and quantitated by chromatography on silicic acid-impregnated paper as previously described (12). A modification in the preparation of the silicic acid-impregnated papers was used: the papers were given a final wash in a 1/4% aqueous solution of Na$_2$EDTA (w/v), air dried, and then stored at 60°C for 24 hr. The presence of the retained Na$_2$EDTA and the mild heating improved the separation and definition of the phospholipid spots obtained after chromatography.

In the early experiments reported here, the total lipid extracts were directly chromatographed on paper. Later in the study, the total lipid extracts were first separated into two fractions before paper chromatography. This was accomplished as follows: about 10 mg of total lipid extract in benzene was applied to a silicic acid column (I.D. 8 mm, 1.5 g of 100 mesh silicic acid). The first fraction was eluted with 20 ml of chloroform-methanol, 4:1 (v/v). This fraction contained the neutral lipids, phosphatidic acid, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and, rarely, small amounts of lecithin. The second fraction was eluted with 20 ml of methanol-water, 99:1 (v/v); it contained lyso-lecithin, sphingomyelin, and lecithin. The column chromatography was carried out at 4°C under nitrogen. This initial fractionation procedure facilitated the complete separation of phosphatidyl inositol and sphingomyelin, and permitted final separation of larger amounts of phospholipid by paper chromatography than was possible when the total extract was chromatographed directly.

4–10 μg of lipid phosphorus was applied to each paper chromatogram. These were developed by ascending chromatography at 4–6°C for 16–18 hr with a solvent consisting of 2,6-dimethyl-4-heptanone, n-butyl ether, acetic acid, and water, 20:20:20:3 (v/v). The phospholipids were identified by their color under ultraviolet light after they had been stained with Rhodamine 6-G, by their reaction to ninhydrin and choline spot tests, and by their relative mobilities, as described previously for this system (12, 13). The individual lipid spots were cut out, and their radioactivity was measured using a low-background, thin window, gas-flow detector system.[1] The

[1] Model C 110-B, Nuclear-Chicago Corporation, Des Plains, Ill.

background was less than 2 cpm with this system which allowed the determination of very low levels of labeling. The lipid phosphorus was eluted from the spots with 0.5 N HCl at 65°C (12). The phosphorus content of the eluates was determined by the method of Bartlett (14). The lipid spots from four to six chromatograms were used to determine each specific activity. Sufficient counts were accumulated so that the standard error of the mean specific activities of sphingomyelin and lecithin (reported below) determined in this way were 5% or less. At least 500 counts were accumulated before deciding that a lipid was not labeled at all. Corrections for physical decay of the ^{32}P were made every 24 hr.

Fig. 1 is a radioautograph of canine bone marrow phospholipids isolated after the in vivo administration of orthophosphate-^{32}P. All of the phospholipids have become labeled and the figure illustrates the separation achieved after paper chromatography of a total lipid extract.

Incubations of RBC with serologically compatible plasma or with dispersed plasma lipids were carried out at 37°C in siliconized or polyethylene flasks, using a
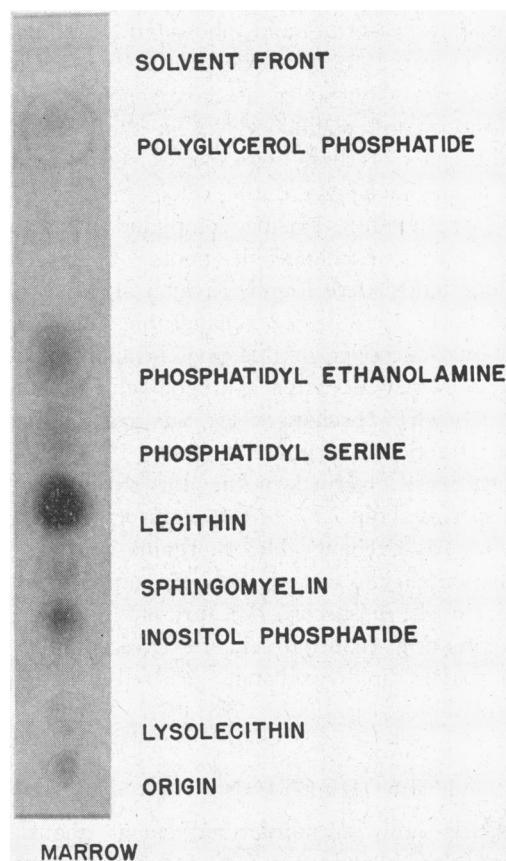

FIGURE 1 Radioautograph of labeled canine bone marrow phospholipids. Orthophosphate-^{32}P (0.15 mc/kg) was administered intravenously, as a single injection, to a dog, and the lipids of the bone marrow cellular elements were isolated 96 hr later. 4.5 μg of total lipid P where chromatographed on silicic acid impregnated paper.

metabolic shaker, for periods of up to 12 hr. Sterile conditions were maintained and were confirmed by culture. Penicillin (100 units/ml) and streptomycin (0.1 mg/ml) were used in some of the incubation and had no effect on the results. Sufficient glucose was added to provide a total concentration of 12 μmoles/ml of blood.

Some of the incubations were carried out with plasma lipids dispersed in an aqueous buffer. The neutral lipids were first removed by silicic acid chromatography and elution with chloroform. The phosphatides were recovered from the column by elution with methanol, and then suspended, with a tissue homogenizer, in a buffer consisting of 5.9×10^{-4} M KH_2PO_4, 2.5×10^{-3} M Na_2HPO_4, 1.4×10^{-1} M NaCl, and 1×10^{-3} M $MgSO_4$, with a resulting pH of 7.4. This suspension of the plasma phosphatides, usually 50 ml in volume, was then subjected to ultrasonic irradiation for 20 min with a dipping probe which generated energy at 20 kc/sec at a power output of 120 watts. The beaker containing the suspension was surrounded by ice water, and the temperature of the suspension remained at 10°C or less during the sonication, although it might have been higher locally, immediately adjacent to the probe. This treatment produced a water-clear solution of the phosphatides, and the concentrations of lecithin and sphingomyelin in the buffer were the same before and after irradiation in each instance. The use of ultrasonic irradiation to produce stable, water-clear dispersions of phospholipids, without breakdown of the compounds, has been described, in detail, by Gammack, Perrin, and Saunders (15). It was not possible to obtain a clear lipid solution if the neutral lipids were not first removed. After the ultrasonic treatment the phospholipid solution was mixed with an equal volume of packed normal erythrocytes from which the buffy coat had been removed. The mixture was incubated for 3 min at room temperature and then centrifuged at 2,000 g for 10 min at 4°C. The supernatant fluid containing the dispersed plasma phospholipids was recovered and then used in test incubations with additional normal erythrocytes. The preliminary absorption procedure served to remove any metallic particles introduced from the probe during the ultrasonic treatment, and to monitor the extent to which the dispersed phospholipids might adhere immediately, in a nonspecific manner, to the red blood cell surfaces. When labeled plasma phospholipids were used no radioactivity was found in the phosphatides of the adsorbing erythrocytes after 3 min, nor in the lipids of the test erythrocytes at time zero.

Plasma was dialyzed against 20–50 volumes of 0.17 M NaCl for 48 hr at 4°C, with three changes of the dialysate. Packed cell volumes were determined by the microhematocrit technique.

RESULTS

When orthophosphate-^{32}P was administered in vivo, the label appeared promptly in the plasma lipid phosphorus; it appeared more slowly and at a lower level in the RBC phospholipids. The general pattern observed in both humans and dogs is shown in Fig. 2. It can be seen that labeled plasma and RBC can be harvested for in vitro studies over a period of days after a single administration of the isotope. Fig. 3 shows the typical labeling
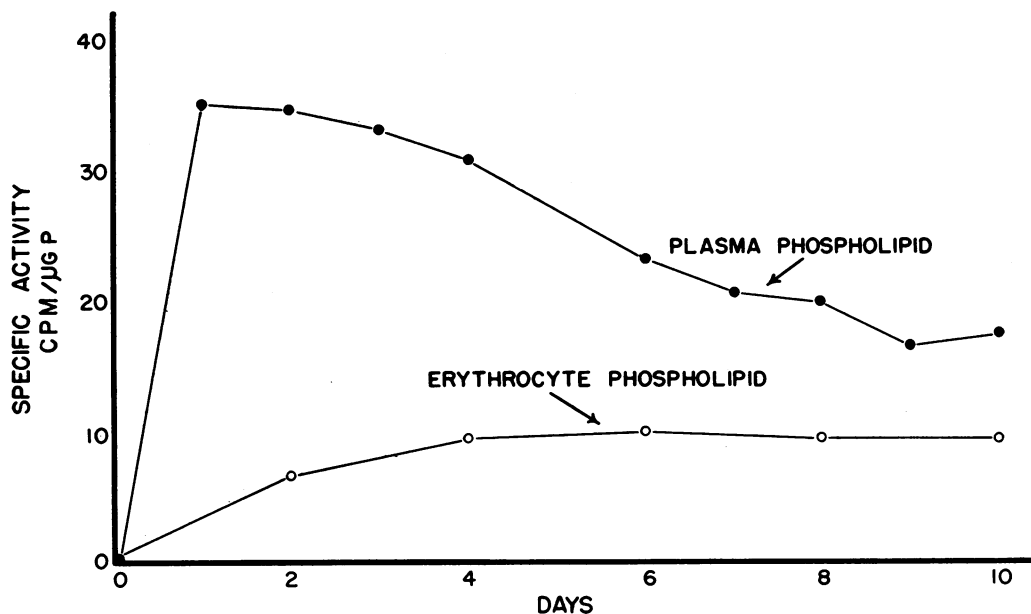
FIGURE 2   Appearance of radioactivity in human plasma and erythrocyte lipid P after ^{32}P administration. Orthophosphate-^{32}P (0.15 mc/kg) was given as a single oral dose. The specific activity of the total plasma and erythrocyte lipid P was determined, as shown, over a period of 10 days.
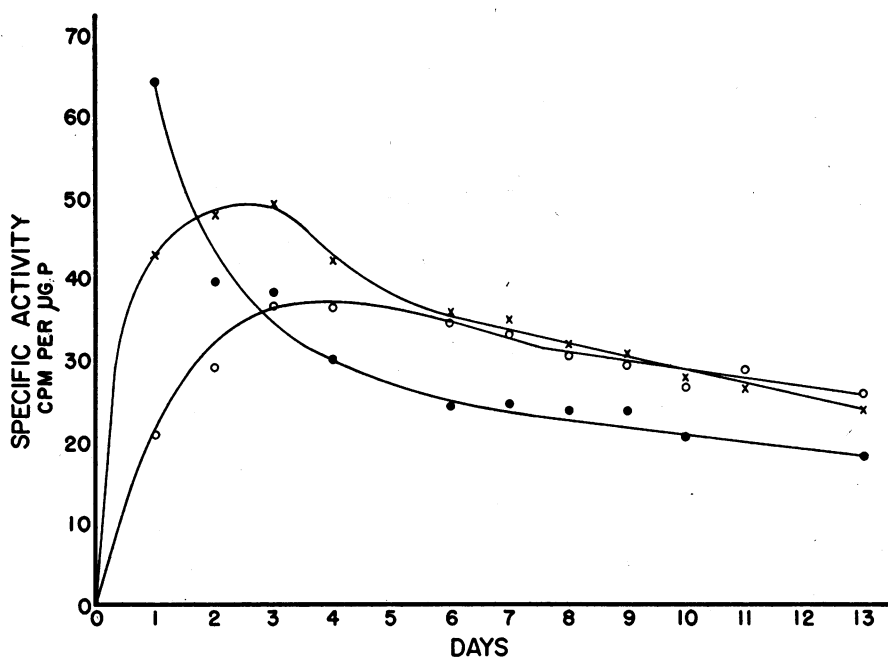
FIGURE 3 Labeling of human plasma P containing compounds after ³²P administration. Orthophosphate-³²P (0.15 mc/kg) was given as a single oral dose. Solid circles = plasma nonlipid P; X's = plasma lecithin; open circles = plasma sphingomyelin.

pattern of plasma sphingomyelin and lecithin (the principal plasma phospholipids, which account for 85–90% of the plasma lipid phosphorus in both species), and of the plasma nonlipid phosphorus, about 90% of which can be removed by dialysis.

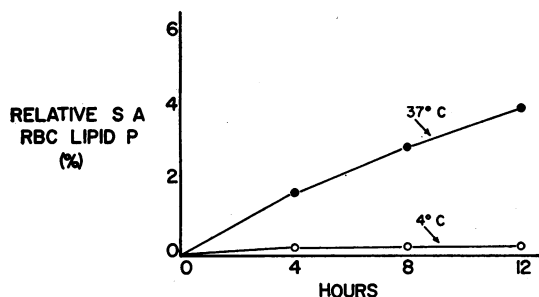The interaction between plasma and erythrocyte lipid phosphorus was studied in vitro by incubating

FIGURE 4 Appearance of labeled plasma P in erythrocyte phospholipids during in vitro incubation. Human labeled plasma was harvested 48 hr after the oral administration of ³²P (0.15 mc/kg) and incubated with serologically compatible normal red blood cells at a hematocrit value of 35%. The specific activity of the total erythrocyte lipid P was divided by the specific activity of the plasma lipid P to obtain the relative specific activity. The results at 37°C are typical of eight such experiments.

serologically compatible labeled plasma with unlabeled RBC at a hematocrit value of 30–40%. Fig. 4 shows that radioactivity appeared in the erythrocyte lipid phosphorus and that it increased at a nearly constant rate for a period of 12 hr, at 37°C. Little radioactivity was found in the erythrocyte lipids when the incubations were carried out at 4°C.

The appearance of radioactivity in the individual major erythrocyte phospholipids was determined at the end of the 12 hr period in incubations carried out at 37°C. The results of these determinations are shown in the first column of Table I. The degree of labeling of erythrocyte lecithin and sphingomyelin is expressed as their "relative specific activities" (RSA). This is defined by the ratio:

$$RSA_{c(t)} = (SA_{c(t)})/(\overline{SA}_p) \qquad (1)$$

where

SA = specific activity, net counts per minute (cpm) per μg of phosphorus

t = duration of incubation (usually 12 hr)

c = individual RBC phospholipid

$\overline{SA}_p$ = average SA of corresponding plasma phospholipid (p): $(SA_{p(t)} + SA_{p(0)})/2$.

TABLE I

RSA of Human Erythrocyte Phospholipids After 12 Hr of Incubation at 37°C in Media Containing ^{32}P*

Erythrocyte phospholipid	Labeled medium			
	Plasma (n = 4)	Dialzed plasma (n = 4)	Plasma phospholipids dispersed by ultra-sonication‡ (n = 5)	Inorganic ^{32}P added to normal plasma (n = 4)
Sphingomyelin	4.0 (3.3–5.2)§	4.0 (3.7–4.1)	4.0 (3.0–4.4)	0
Lecithin	8.0 (7.5–9.5)	9.0 (8.5–9.4)	8.0 (6.8–9.2)	0
Phosphatidyl serine	0	0	0	0
Phosphatidyl ethanolamine	0	0	0	0

* Abbreviation: RSA, relative specific activity.

‡ At 6 hr, (n = 3), lecithin RSA = 4.0 (3.8–5.1), sphingomyelin RSA = 2.0 (1.2–2.2).

§ Mean values, ranges in parentheses.

This ratio can be expressed as a percentage by multiplying the RSA by 100. In each instance, $SA_{c(0)}$ was zero. The SA of plasma sphingomyelin and lecithin decreased very little during these experiments; they were approximately 95% of the time zero values for both lipids in each case at the end of 12 hr. Thus, SA_p was virtually constant. Column 2 of Table I shows that prior removal by dialysis of the plasma nonlipid phosphorus had no effect on the labeling achieved by erythrocyte lecithin and sphingomyelin. Column 3 of Table I shows that labeled plasma phospholipids, dispersed in an aqueous buffer at approximately the same concentration as they were present in native plasma, produced the same degree of labeling of the erythrocyte phospholipids as was found with whole plasma. Column 4 of the table shows that when orthophosphate-^{32}P was added to the incubation mixtures as the only source of isotope there was no incorporation of radioactivity in any of the four major erythrocyte phosphatides over a period of 12 hr. All of these results indicate that plasma dialyzable phosphorus and inorganic phosphorus play no part in the labeling of the major erythrocyte phospholipids and that the plasma phosphatides are the sole immediate precursors in the labeling of the corresponding erythrocyte compounds.

The relative specific activities of erythrocyte lecithin and sphingomyelin shown in Table I are small (less than 10%) and, therefore, these product-precursor specific activity ratios are a good approximation of the actual fractional turnover per 12 hr of the cell phospholipids in the exchange process (16). After 12 hr the recovery of each of the two plasma and four erythrocyte lipids measured averaged 100% (with a range of 97–103%). Thus, there was no measurable net movement of phospholipids between plasma and erythrocytes in these studies. We have previously reported that in the presence of adequate glucose normal erythrocytes lose very little lipid when they are incubated under the same circumstances as here for periods up to 24 hr (17).[2]

Measurable radioactivity was not found in erythrocyte phosphatidyl serine and phosphatidyl ethanolamine, probably because these two lipids are almost absent from the plasma. In two experiments, not included in column 3 of Table I, labeled plasma lecithin virtually free of sphingomyelin was obtained by silicic acid chromatography. When this material was dispersed by ultrasonication and incubated with RBC, the resulting RSA of the erythrocyte lecithin, after 12 hr, was 7.8% and 8.3%, a normal degree of labeling. Erythrocyte sphingomyelin, on the other hand, was not labeled at all in these two experiments. These results suggest that the plasma-erythrocyte lipid exchange is, in fact, between corresponding compounds, and that phosphoryl choline, present in both lecithin and sphingomyelin, does not exchange independently of the entire lipid molecule in which it is present.

The exchange of lecithin and sphingomyelin between plasma and erythrocytes was also studied by incubating cells which had become labeled in vivo (harvested 3–6 days after the initial administration of orthophosphate-^{32}P, Fig. 2) with unlabeled plasma. In these experiments, labeling of the plasma lipid phosphorus increased at a constant rate for 12 hr and this period was, again,

[2] Over a period of 12 hr and using Na₂EDTA plasma in nonwettable surfaces, we found no evidence for the activity of the serum cholesterol-esterifying enzyme first described by Sperry (18). In the presence of glass surfaces and over longer periods of incubation a reduction of serum lecithin and an increased amount of esterified cholesterol results from the activation of this enzyme (18, 19).

chosen for analyzing the SA of the individual phosphatides.

It was possible to calculate the movement of RBC lecithin and sphingomyelin into the plasma from these experiments. The RSA's of the "product" compounds, plasma lecithin and sphingomyelin, were determined and this gave the fraction of the plasma lipid derived from the corresponding erythrocyte lipids at 12 hr. This value was multiplied by the total amount of the plasma lipid per ml of incubation mixture which gave the absolute amount of the lipid moving from the erythrocyte to the plasma compartment. This product was then divided by the amount of the lipid in the erythrocyte compartment, and the resulting quotient gave the fractional turnover of the erythrocyte lipid per 12 hr, measured in the outward direction. Combining the three steps, the expression for this calculation is:

$$SA_{p(t)}/\overline{SA}_c \times \mu g_p \times 1/\mu g_c \qquad (2)$$

where

$\mu g_p = \mu g$ of individual plasma lipid phosphorus per ml of incubation mixture

$\mu g_c = \mu g$ of corresponding RBC lipid phosphorus per ml of incubation mixture, and the other symbols as in expression 1.

$SA_{p(0)}$, again, was zero, and, as mentioned, μg_p and μg_c did not change. The results of these outward studies are shown in the first column of Table II. It can be seen (Table II) that the fractional exchange rates and actual flows of erythrocyte sphingomyelin and lecithin calculated from both types of in vitro experiments do not agree, and that the rates determined from the outward studies are considerably larger.

No net movement of lipid phosphorus between the erythrocyte and plasma compartments was discernible in these experiments, however, and the rates of actual lipid exchange should, therefore, be equal in both directions. The discrepancy between the calculated inward and outward rates may be resolved by assuming that the radioactivity in human erythrocyte lecithin and sphingomyelin, labeled in vivo, was not distributed uniformly throughout these lipid pools, but that it was distributed in only 63% of the lecithin and 29% of the sphingomyelin. If this is so, calculation of the fractional turnover from the outward experiment would require that $\overline{SA}_c$ of expression 2, (the SA of the total erythrocyte lecithin or sphingomyelin, which is what can be measured) be divided by approximately 0.6 and 0.3, respectively, to yield the true SA of the erythrocyte molecules actually participating in the lipid movement. The calculated outward rate would then be equal to the observed inward rate. This hypothesis postulates that only about 60% of human erythrocyte lecithin and 30% of the sphingomyelin are actually capable of exchange with the corresponding plasma compounds. As will be shown below, results of the in vivo studies of lipid exchange over a period of days support this view.

Parallel in vitro studies were carried out with canine blood. The results are shown in Table III. The overall results were quite comparable to those

TABLE II
*12-Hr Exchange Rates of Human Erythrocyte Sphingomyelin and Lecithin Determined In Vitro**

RBC lipid	Outward labeled RBC and unlabeled plasma (n = 4)		Inward‡ labeled RBC and unlabeled plasma (n = 4)		Ratio inward/ outward
	Fractional turnover	μmoles per ml RBC $\times 10^2$	Fractional turnover	μmoles per ml RBC $\times 10^2$	
Sphingomyelin	% 14.0 (12.5–15.8)§	14.0	% 4.0 (3.3–5.2)	4.0	0.29
Lecithin	13.0 (12.5–13.8)	17.5	8.0 (7.5–9.5)	11.0	0.63

* Abbrevaition: RBC, red blood cell.
‡ Values from column 1 of Table I.
§ Mean values, ranges in parentheses.

TABLE III

*12-Hr Exchange Rates of Canine Erythrocyte Sphingomyelin and Lecithin Determind In Vitro**

| RBC lipid | Outward labeled RBC and unlabeled plasma (n × 4) | | Inward labeled plasma and unlabeled RBC (n = 4) | | Ratio inward/ outward |
	Fractional turnover	μmoles per ml RBC × 10²	Fractional turnover	μmoles per ml RBC × 10²	
	%		%		
Sphingomyelin	10.0 (8.6–12.1)‡	7.0	7.6 (6.3–8.9)	5.3	0.76
Lecithin	10.0 (8.8–12.0)	18.0	6.2 (5.4–7.1)	11.2	0.62

* Abbreviations: RBC, red blood cell.
‡ Mean values, ranges in parentheses.

obtained with the human material. Phosphatidyl serine and phosphatidyl ethanolamine were stable; sphingomyelin and lecithin were found to undergo exchange between the plasma and erythrocytes. The major difference found was that the size of the exchangeable sphingomyelin erythrocyte pool was larger in the dog. The distribution of sphingomyelin between plasma and erythrocytes is the same in both species, the plasma-erythrocyte molar ratio being approximately 1 : 1. The observed difference may, therefore, be due to differences in the distribution and binding of sphingomyelin within the membrane in the two species.

The exchange process was also studied in vivo. Serial determinations of the SA of erythrocyte and plasma phospholipids were carried out in five patients and four dogs after the administration of orthophosphate-^{32}P. Four of the humans and one dog received a single dose of the isotope. The other subjects received ^{32}P in divided doses.

When the isotope was administered as an initial loading dose followed by small daily replacements,
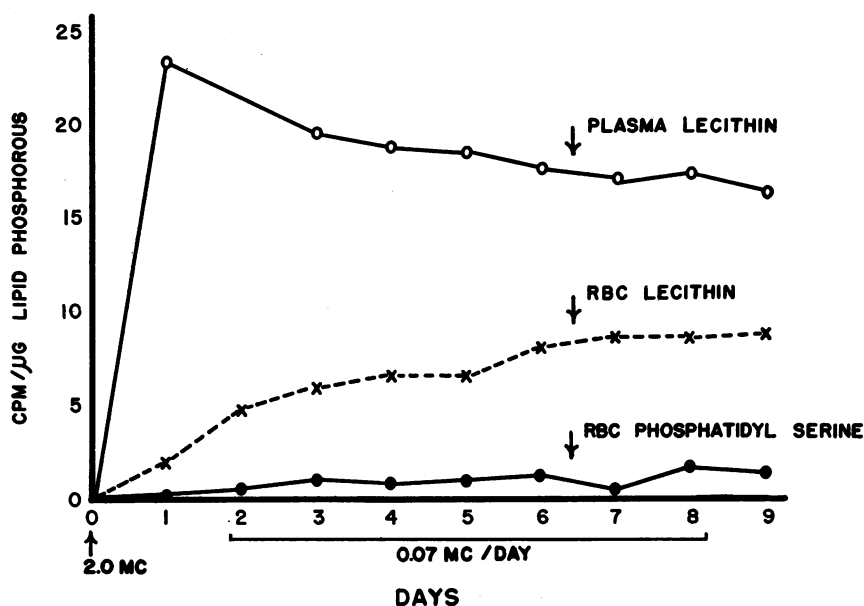

FIGURE 5 Appearance of radioactivity in some individual phospholipids of canine blood after ^{32}P administration. The isotope was administered intravenously (0.15 mc/kg) in divided doses, as indicated.

the SA of plasma lecithin and sphingomyelin could be kept nearly constant at a relatively high level for a period of days. Fig. 5 shows the SA of plasma lecithin and erythrocyte phosphatidyl serine and lecithin observed in a typical experiment in the dog when the isotope was administered in divided doses. The basic features of the exchange process seen in the in vitro studies are apparent in this type of experiment. The SA of plasma lecithin remained considerably higher than that of the RBC compounds, and it remained relatively constant from the 2nd to the 9th day. The SA of erythrocyte lecithin increased until about the 6th day and achieved a level which was 50–60% of the plasma lecithin SA. These findings suggest, qualitatively, that some fraction of the circulating erythrocyte lecithin pool exchanges very slowiy or not at all with plasma lecithin, as was indicated by the in vitro studies. The SA of phosphatidyl serine remained very low and was not measurable during the first 2 or 3 days. The labeling of this compound may be due to the influx of new cells from the bone marrow where phospholipid synthesis occurs (Fig. 1).

In all the in vivo experiments a quantitative approximation of the fraction of erythrocyte lecithin or sphingomyelin which existed as labeled lipid on any given day was obtained by dividing the SA of the erythrocyte lipid on that day by the weighted average of the SA of the corresponding plasma lipid, to that point, according to the following expression:

$$Y_{c(k)} = \frac{SA_{c(k)}}{\dfrac{\sum\limits_{i=0}^{k} SA_{p(i)}}{k}} \qquad (3)$$

where

$Y_{c(k)}$ = fraction of individual RBC lipid pool which exists as labeled lipid on the kth day

$SA_{c(k)}$ = specific activity of the RBC lipid on the kth day

$\sum\limits_{i=0}^{k} SA_{p(i)}$ = sum of the specific activities of the corresponding plasma lipid from day zero to the kth day.

The fraction of the erythrocyte sphingomyelin or lecithin pools which are labeled (that is, the fraction of the erythrocyte lipids that was at one time in the plasma) can also be predicted exactly

from the fractional turnover and sizes of the exchangeable erythrocyte pools determined in the in vitro studies, (Table IV), by using this expression [3]:

$$Y_{c(t)} = (1 - e^{-rt}) \times E \qquad (4)$$

where

t = time (12 hr = 1 unit)

$Y_{c(t)}$ = fraction of the total individual RBC lipid pool which exists as labeled lipid at time t

r = 12 hr fractional turnover rate of the exchangeable pool of the RBC lipid (Table IV)

E = fractional size of the exchangeable pool of the RBC lipid

e = base of the natural logarithms.

The observed results for $Y_{c(k)}$ and the predicted values were compared at each point in the five human subjects and the four dogs studied for periods of up to 15 days. All of these results are shown in Fig. 6. It can be seen that the observed data agree well with the solid curves of the figure. These solid curves indicate the expected labeling of the erythrocyte lipids if the existence and size of exchangeable lecithin and sphingomyelin pools and their fractional turnover rate, determined in vitro and given in Table IV, are correct. The broken curves, as described in the legend of Fig 6, show the labeling of the erythrocyte compounds

[3] In expression 3 1 day (k) is used as the unit of time because the SA of the plasma and RBC lipids were determined daily in the in vivo studies; 12 hr (t) is used in expression 4 because "r" was determined over this period in vitro. Expressions 3 and 4 are minor adaptions of the calculation of turnover from a precursor with constant SA given by Zilversmit (16). Expression 3 will give a good approximation of $Y_{c(k)}$ if SA_p is relatively constant and if SA_c does not decrease over the time interval studied.
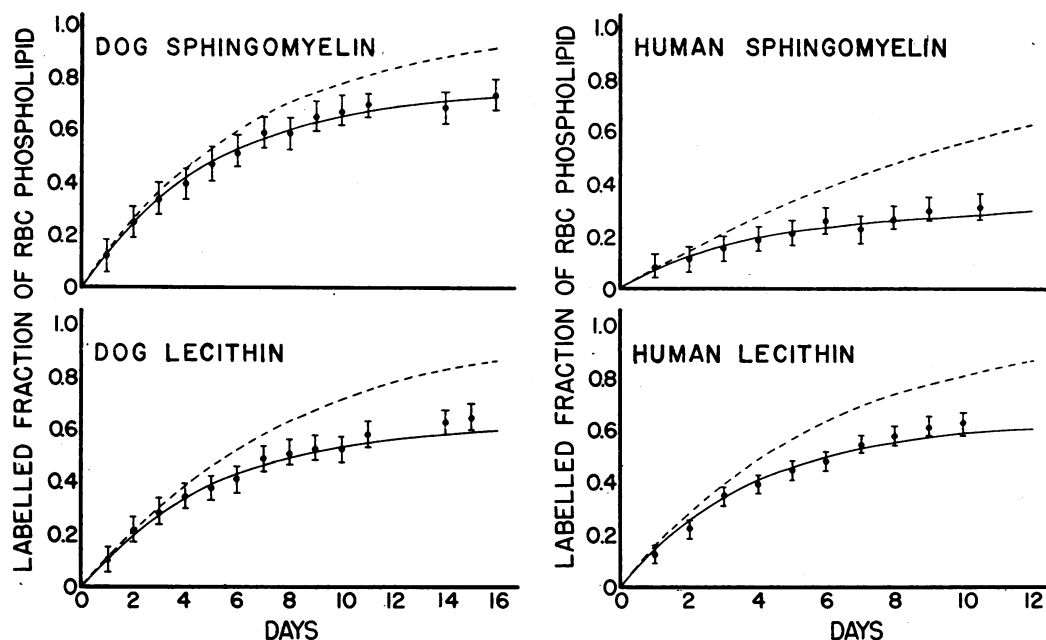
FIGURE 6 Comparison of the observed in vivo labeling of erythrocyte sphingomyelin and lecithin with that predicted from the in vitro data. The solid curves were constructed from expression 4 of the text with the values for "E" and "r" given in Table IV. The broken curves were contructed from, simply, the expression $Y_{c(t)} = 1 - e^{-rt}$, where "r" is the fractional influxes observed in vitro and given in Tables II and III. This calculation assumes that the cellular lipids are entirely exchangeable. Bracketed solid circles mean ± 2 SE of the observed labeling, obtained from expression 3 of the text, in five humans and four dogs.

that would have been obtained in these experiments if all the cellular lecithin and sphingomyelin were exchangeable. The presence of exchangeable pools of erythrocyte lecithin and sphingomyelin will affect the observed labeling of these compounds primarily by altering the loss back to the plasma of newly acquired label during a given time interval. This loss of acquired label will be greater if exchangeable and nonexchangeable pools of a given lipid exist. When the RSA of the cellular lipids is low (less than 20%) this greater loss is a small factor, as indicated in Fig. 6 by the close coincidence, for 3 to 4 days, of the broken and solid curves. After this period, the greater loss of newly acquired label when a nonexchangeable pool is present becomes increasingly significant and the two curves diverge progressively. The good agreement beyond 4 days of the observed labeling with the solid curves of Fig. 6 strongly suggests, therefore, that exchangeable and nonexchangeable pools, of the approximate sizes postulated from the in vitro results, do, in fact, exist. The in vivo results also support the validity of

the fractional turnover rates determined in vitro. It should be mentioned, however, that although the above hypotheses satisfactorily account for all the results, this does not completely exclude the possibility that more than two membrane compartments actually exist for each phospholipid; however, the overall labeling of the system is reasonably well predicted by the two compartment membrane model.

Two factors, in addition to phospholipid exchange, will affect the labeling of erythrocyte sphingomyelin and lecithin in vivo. (a) The influx of new erythrocytes into the circulation. These new cells will contain lipids synthesized from simple precursors (including the administered inorganic ^{32}P) in the bone marrow. After 3–4 days this mechanism will tend to raise the observed labeling of erythrocyte lecithin and sphingomyelin above the value predicted from exchange alone. In one experiment in the dog, the bone marrow cellular elements were isolated 3 days after the administration of ^{32}P. Identical SA's were found in the four major phospholipids. This suggests

that the low SA observed in phosphatidyl serine shown in Fig. 5 may serve as a rough guide to the effect of new cell synthesis, and that this is negligible for 6–9 days. (*b*) Removal of senescent erythrocytes from the circulation. About 1% per day of the circulating RBC mass will be removed this way in both man and the dog. This will tend to lower the observed labeling of circulating erythrocyte lecithin and sphingomyelin, but this effect should not be noticeable for 6–10 days. The fact that there was little deviation from the observed values and those predicted on the basis of exchange alone for as long as 15 days (Fig. 6) suggests that the opposite effects of new cell production and red cell senescence may tend to cancel each other.

DISCUSSION

In the present work some of the quantitative aspects of the exchange between erythrocyte lecithin and sphingomyelin with the corresponding plasma compounds have been described in man and the dog. These findings are summarized in Table IV. Exchangeable and nonexchangeable fractions of each of these two cellular lipids were found in both species. Rowe (8) has previously suggested that blood cellular lecithin, in humans, does not exchange completely with plasma lecithin.

It can be seen from Table IV that the turnover times of the exchangeable pools of erythrocyte sphingomyelin and lecithin, in both species, are approximately 5 days. Thus, alterations in the fatty acid composition of the plasma compounds can affect, in a very significant manner, the corresponding erythrocyte membrane lipids during the life span of a given red blood cell. The actual amounts of lipid involved in the exchange process, however, are quite small. In the case of lecithin, in both species studied here, the exchange is about 9×10^{-3} mmoles/liter of erythrocytes per hour. This is in contrast to the active transport of monovalent cations across the human erythrocyte membrane, for example, which is about 3 mmoles/liter of erythrocytes per hour.

In the present study, by using ^{32}P to evaluate turnover, it was found that erythrocyte phosphatidyl serine was completely stable and phosphatidyl ethanolamine nearly so. As mentioned above, this may simply result from the virtual absence of these two phospholipids from the plasma of man

and the dog. Erythrocyte cephalins have been found to be stable in exchange studies with ^{32}P in the rat (10) where these compounds are also nearly absent from plasma. The different rates and degree of exchangeability found for sphingomyelin and lecithin, particularly in the human, on the other hand, suggest that, in addition to plasma concentration, the position of a lipid molecule in the membrane and the nature of its bond to membrane protein (ionic vs. hydrophobic) may also influence its exchange. Cholesterol, for example, is thought to be bound in many membranes only to the hydrophobic portion of phospholipids, primarily by the relatively weak van der Waals forces (20). This erythrocyte lipid has been found to exchange very rapidly with plasma free cholesterol (turnover time less than 12 hr) in both man and the dog (21–23). This is in contrast to the much slower rate of phospholipid exchange described here. The latter compounds probably are bound to membrane protein by charge-charge interactions, in large part (20).

Inorganic ^{32}P was not incorporated into any of the four major erythrocyte phospholipids. In these compounds, the phosphorus group exists as a diester and is internally placed in the molecule. Incorporation of inorganic ^{32}P would, therefore, require enzymatic breakdown and resynthesis of the lipid. There is a substantial body of evidence, summarized by van Deenen and de Gier (2), that mature nonnucleated erythrocytes are incapable of carrying out such synthetic lipid processes. For this reason, and because, as mentioned above, phosphoryl choline from lecithin did not appear in erythrocyte sphingomyelin, we believe that the exchange of lipid phosphorus herein described probably involves the entire lipid molecule.

Very small amounts of lysolecithin were found in the plasma and erythrocytes of both species studied. This compound was weakly labeled and its participation in the exchange process could not be evaluated. In the rat, on the other hand, substantial amounts of lysolecithin have been reported in the plasma and this compound is exchanged actively (9–11). Mulder and coworkers (24, 25) and, more recently, Tarlov (26) have indicated that reversible acylation by plasma fatty acids of membrane lysolecithin, a process known to occur in human red blood cells (27, 28), accounts for a substantial proportion of lecithin turn-

over in rat erythrocytes. The exact degree to which such transacylation of erythrocyte phospholipids occurs in human is not known at present. As suggested by Farquahar and Ahrens (29), additional studies using phospholipids labeled with both ^{32}P and ^{14}C are needed to compare the contributions of transacylation and exchange of entire molecules to the dynamic state of the erythrocyte phospholipids.

The nonsymmetrical exchange of erythrocyte phospholipids described here is in contrast to the process of cellular lipid loss which has been reported during the course of in vivo ageing (30, 31), during in vitro incubations of abnormal cells for 24 hr (17), and in normal cells incubated for more prolonged periods (32). In these instances, the loss of lipid has been found to involve all lipid compounds equally. The concomitant formation of small membrane fragments of varying sizes also observed in some of these studies (33) provided an explanation for the symmetrical nature of the lipid loss. The process of lipid exchange may, however, prove important in the pathogenesis of erythrocyte abnormalities associated with alterations in plasma lipids, as has been suggested previously (2, 26, 34). It is hoped that the quantitative data on phospholipid exchange given in the present study may prove helpful in further exploration of this possibility.

ACKNOWLEDGMENT

REFERENCES

1. Reed, C. F. 1959. Studies of in vivo and in vitro exchange of erythrocyte and plasma phospholipids. J. Clin. Invest. 38: 1032. (Abstr.)
2. van Deenen, L. L. M., and J. de Gier. 1964. Chemical composition and metabolism of lipids in red cells of various animal species. In The Red Blood Cell. C. Bishop and D. M. Surgenor, editors. Academic Press, Inc., New York. 7: 243.
3. London, I. M. 1960. The metabolism of the erythrocyte. Harvey Lectures. 56: 151.
4. Muir, H. M., A. Neuberger, and J. C. Perrone. 1952. Further isotopic studies on haemoglobin formation in the rat and rabbit. Biochem, J. 52: 87.
5. Hevesy, G., and L. Hahn. 1940. Turnover of lecithin, cephalin and sphingomyelin. Kgl. Danske Videnskab. Selskab. Biol. Med. 15: 1.

6. Hahn, L., and G. Hevesey. 1939. Interaction between the phosphatides of the plasma and corpuscles. Nature. 144: 72.
7. Lovelock, J. E., A. T. James, and C. E. Rowe. 1960. Lipids of whole blood. 2. The exchange of lipids between the cellular constituents and the lipoproteins of human blood. Biochem. J. 74: 137.
8. Rowe, C. E. 1960. The phospholipids of human-blood plasma and their exchange with the cells. Biochem. J. 76: 471.
9. Polonovski, J., and M. Paysant. 1963. Métabolism phospholipidique du sang. VIII. Échange des phospholipides marqués entre globules et plasma sanguin in vitro. Bull. Soc. Chim. Biol. 45: 339.
10. Sakagami, T., O. Minari, and T. Orii. 1965. Interaction of individual phospholipids between rat plasma and erythrocytes in vitro. Biochim. Biophys. Acta. 98: 356.
11. Mulder, E., and L. L. M. van Deenen. 1965. Metabolism of red-cell lipids. III. Pathways for phospholipid renewal. Biochim. Biophys. Acta. 106: 348.
12. Reed, C. F., S. N. Swisher, G. V. Marinetti, and E. G. Eden. 1960. Studies of the lipids of the erythrocyte. I. Quantitative analysis of the lipids of normal human red cells. J. Lab. Clin. Med. 56: 281.
13. Marinetti, G. V., J. Erbland, and J. Kochen. 1957. Quantitative chromatography of phosphatides. Fed. Proc. 16: 837.
14. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234: 466.
15. Gammack, D. B., J. H. Perrin, and L. Saunders. 1964. The dispersion of cerebral lipids in aqueous media by ultrasonic irradiation. Biochim. Biophys. Acta. 84: 576.
16. Zilversmit, D. B. 1960. The design and analysis of isotope experiments. Am. J. Med. 29: 832.
17. Reed, C. F., and S. N. Swisher. 1966. Erythrocyte lipid loss in hereditary spherocytosis. J. Clin. Invest. 45: 777.
18. Sperry, W. M. 1935. Cholesterol esterase in blood. J. Biol. Chem. 111: 467.
19. Glomset, J. A., F. Parker, M. Tjaden, and R. H. Williams. 1962. The esterification in vitro of free cholesterol in human and rat plasma. Biochim. Biophys. Acta. 58: 398.
20. van Deenen, L. L. M. 1965. Phospholipids and biomembranes. In Progress in the Chemistry of Fats and Other Lipids. R. T. Holman, editor. Pergamon Press Ltd., Oxford. 8(Pt. 1): 1.
21. Hagerman, J. S., and R. G. Gould. 1951. The in vitro interchange of cholesterol between plasma and red cells. Proc. Soc. Exptl. Biol. Med. 78: 329.
22. London, I. M., and H. J. Schwarz. 1953. Erythrocyte metabolism. The metabolic behavior of the cholesterol of human erythrocytes. J. Clin. Invest. 32: 1248.
23. Eckles, N. E., C. B. Taylor, D. J. Campbell, and R. G. Gould. 1955. The origin of plasma cholesterol and the rates of equilibration of liver, plasma, and erythrocyte cholesterol. J. Lab. Clin. Med. 46: 359.
24. Mulder, E., and L. L. M. van Deenen. 1965. Metabolism of red-cell lipids. I. Incorporation in vitro of

fatty acids into phospholipids from mature erythrocytes. *Biochim. Biophys. Acta.* **106**: 106.

25. Mulder, E., J. W. O. vandenBerg, and L. L. M. van Deenen. 1965. Metabolism of red-cell lipids. II. Conversions of lysophosphoglycerides. *Biochim. Biophys. Acta.* **106**: 118.

26. Tarlov, A. R. 1966. Lecithin and lysolecithin metabolism in rat erythrocyte membranes. *Blood.* **28**: 990. (Abstr.)

27. Oliveira, M. M., and M. Vaughan. 1964. Incorporation of fatty acids into phospholipids of erythrocyte membranes. *J. Lipid Res.* **5**: 156.

28. Robertson, A. F., and W. E. M. Lands. 1964. Metabolism of phospholipids in normal and spherocytic human erythrocytes. *J. Lipid Res.* **5**: 88.

29. Farquhar, J. W., and E. H. Ahrens, Jr. 1963. Effects of dietary fats on human erythrocyte fatty acid patterns. *J. Clin. Invest.* **42**: 675.

30. Westerman, M. P., L. E. Pierce, and W. N. Jensen. 1963. Erythrocyte lipids: a comparison of normal young and normal old populations. *J. Lab. Clin. Med.* **62**: 394.

31. van Gastel, C., D. vandenBerg, J. de Gier, and L. L. M. van Deenen. 1965. Some lipid characteristics of normal red blood cells of different age. *Brit. J. Haemat.* **11**: 193.

32. Weed, R. I., and C. F. Reed. 1966. Membrane alterations leading to red cell destruction. *Am. J. Med.* **41**: 681.

33. Weed, R. I., and A. J. Bowdler. 1966. Metabolic dependence of the critical hemolytic volume of human erythrocytes: relationship to osmotic fragility and autohemolysis in hereditary spherocytosis and normal red cells. *J. Clin. Invest.* **45**: 1137.

34. Ways, P., and C. F. Reed. 1965 The nature and dynamic behavior of red cell lipids. *Scand. J. Haematol.* **10**: 34.