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THE EXCHANGE OF ERYTHROCYTE MEMBRANE PHOSPHOLIPIDS WITH RAT LIVER EXTRACTS IN VITRO

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Intact rat or human erythrocytes and their isolated (ghost) membranes were incubated with the high speed supernatant fraction of homogenates derived from ^{32}P-labeled rat livers. Phospholipid molecules were transferred between the red cell membranes and the liver extracts, as reflected by the convergence of their specific radioactivities with time. Whereas ghosts usually approached isotopic equilibrium with the liver supernatant fraction during a few hours of incubation at 37°C, the exchange of phospholipids by intact cells was no more than one-half, even after 18 hr. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin were all exchanged in both intact cells and ghosts, albeit to different extents. (A control experiment, incubating ^{32}P-labeled rat erythrocytes or ghosts with unlabeled rat liver extracts, also demonstrated the exchange of all four major phospholipids.) These data may signify that the phospholipids on the cytoplasmic side of the membrane of intact erythrocytes do not exchange with the phospholipids in exogenous liver extracts. If so, all four major phospholipid classes would appear to be present to some extent at both membrane surfaces. The first inference is in agreement with several other studies on this membrane, while the second inference is not.

An absolute asymmetry in the orientation of the proteins at the two surfaces of human erythrocyte membranes has been suggested by a variety of recent studies (reviewed in Ref. 1). Glycolipids likewise appear to be confined exclusively to the outer membrane surface (2). Probing phospholipid accessibility in intact cells, unsealed ghosts, and sealed vesicles with specific antisera (3), covalent ligands (4,5), and lipolytic enzymes (6–9) has led to the hypothesis that the four major phospholipid classes may also be unequally disposed in this membrane, with phosphatidylcholine (PC) and sphingomyelin (SM) found principally at the outer membrane surface and phosphatidylethanolamine (PE) and phosphatidylserine (PS) at the cytoplasmic side.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; 5P8 buffer, 5 mM sodium phosphate (pH 8.0); RBC, red blood cell; extract, the supernatant fraction from rat liver homogenates after centrifugation at $10^5 \times g$ for 1 hr; Q, the ratio of the phospholipid specific activity at equilibrium in the membrane fraction to that of the corresponding supernatant fraction (see text).

We have now investigated the accessibility of erythrocyte membrane phospholipids by a different means: their ability to undergo exchange with their counterparts in rat liver extracts. The 105,000 × g supernatant fraction of rat liver homogenates contains minute membranous vesicles (10) and soluble proteins which stimulate the exchange of membrane phospholipids, made radioactive by the in vivo administration of $Na_2H^{32}PO_4$, with mitochondria (11) and a variety of other natural and synthetic membrane species (see, for review, Refs. 12 and 13). Our data suggest that the entire phospholipid pool in ghosts can exchange with rat liver extracts, while intact cells exchange at most about one-half of their phospholipids. Since all four major phospholipid classes were exchanged to some extent by intact red cells, each phospholipid class appears to be represented in part at the external erythrocyte membrane surface.

METHODS

$Na_2H^{32}PO_4$ was obtained from Schwarz/Mann or New England Nuclear Corp., carrier-free in 0.01 M HCl. All inorganic chemicals and organic solvents were reagent grade or better, from Fisher, Mallinckrodt, or Baker.

^{32}P-labeled Rat Liver Extracts and Erythrocytes

Pairs of 300–400 gm male Sprague Dawley rats were given approximately 1 ml of 20 mM Na_2HPO_4, containing 750 μCi of ^{32}P, by intraperitoneal injection. After 16–18 hours, the animals were anesthetized with Nembutal and their livers excised, chilled, and minced. Unless indicated otherwise, all subsequent procedures were performed at 0–4°C. Each gram of liver was homogenized in a Potter-Elvehjem hand homogenizer in 2 ml of a 0.25 M sucrose–1 mM EDTA solution, as previously described (11). The extract was centrifuged at 2000 rpm for 10 min (IEC B-20 Rotor), at 15,000 rpm for 30 min (IEC A-147 Rotor), and at 39,000 rpm for 1 hr in a Spinco model 40 Rotor. (In a few cases, the last centrifugation was repeated a second time, without altering the outcome of the experiment.) The supernatant fraction was stored at 5°C or −20°C overnight and centrifuged for 15 min at 15,000 rpm immediately prior to use to remove any aggregates which might have arisen during storage.

In some experiments, erythrocytes were harvested from the aorta of rats injected with ^{32}P for exchange against unlabeled liver extracts.

Erythrocytes and Membranes

Rat erythrocytes were obtained from the aorta of anesthetized 300–400 gm male Sprague Dawley rats, using oxalate to forestall clotting. The blood was usually collected the evening before use and stored at 5°C. Normal human blood was either freshly drawn into 10 mM EDTA or was out-dated from the blood bank. Red cells were washed 3 times with 150 mM NaCl–5P8.

Unsealed human red cell ghosts were prepared by hemolysis and washing in 40 vol of ice-cold 5P8 (14). This procedure could not be applied to rat red blood cells because their hemoglobin precipitates in the chilled, low ionic strength buffer. Therefore, rat erythrocytes were hemolyzed in 40 vol of 0.15 M NH_4HCO_3, which causes Donnan-osmotic swelling, lysis, and spontaneous resealing of the membranes (cf. Ref. 15). These ghosts were then pelleted and washed twice more with 5P8 as above to prepare unsealed, hemoglobin-free ghosts.

Exchange Reactions

Packed intact erythrocytes (0.5 ml) or ghosts were mixed with 1.5 ml of liver extract (one or the other component bearing the ^{32}P label) and incubated in conical centrifuge tubes in a shaking water bath for 0–18 hr at 37°C. The reaction was terminated by chilling on ice and centrifuging the erythrocytes (at 2,000 rpm for 10 min) or ghosts (at 15,000 rpm for 10 min) from suspension. Of the liver supernatant extract 1.2 gm were aspirated from each tube with Pasteur pipettes; the pellets were resuspended and washed 3 times in 5P8 (ghosts) or 0.15 M NaCl in 5P8 (red cells). The cells were then hemolyzed, and ghost membranes prepared as described above.

Phospholipid Analysis

Specific activity in the four major phospholipid classes was determined as follows. Duplicate aliquots of each sample were extracted thoroughly with 20 vol of chloroform: methanol (2:1, vol/vol) at room temperature (16). The organic phase was washed successively with 1 vol of 2 M $MgCl_2$ and 2 vol of water, then dried under a stream of nitrogen at 40°C. The residue was dissolved in 2.0 gm chloroform:methanol (2:1), and 0.60 gm of this solution (approximately 1 μmole phospholipid) was applied to a thin layer chromatography plate (silica gel H, 20/20, Analtech, Inc.). Two dimensional chromatography was performed by a modification (A.R. Tarlov and M.S. Gibson, unpublished) of the method of Abramson and Blecher (17).

The four major phospholipid spots, visualized with I_2, were scraped into test tubes. The phospholipids were eluted into methanol:formic acid:water (420:60:20, by volume) and dried under an N_2 stream at 40°C. Each sample was dissolved in 1.0 ml of 10% formic acid in methanol. From each sample, 0.6 ml aliquots were dried and assayed for phosphorous by the method of Bartlett (18). Next, 0.3 ml aliquots were transferred to scintillation vials. To each vial was added 4.7 ml of methanol and 10 ml of toluene containing 4 gm of 2,5-diphenyloxazole (PPO) and 0.2 gm of 1,4-bis[5-phenyloxazolyl]benzene (POPOP) per liter. Samples were counted in a Packard scintillation counter. All data reported are the averages of duplicate samples, each of which was analyzed in duplicate.

RESULTS

Experimental Conditions

The four principal phospholipids present in rat and human erythrocyte membranes (19) were found in rat liver extracts in these relative proportions (percent ± SEM for 17 determinations): SM, 7.8 ± 0.43; PC, 65.6 ± 1.1; PE, 12.1 ± 0.81; PS, 14.5 ± 1.1. The phospholipid content of 1.5 ml of liver extract was approximately half that of the 0.5 ml of red cells or ghosts with which it was incubated. Enough of each phospholipid was therefore present in the liver extract and red cell membranes for isotope exchange to be readily measurable.

The protocol employed for phospholipid exchange was adapted from that of Wirtz and Zilversmit (11). Care was taken to remove in advance all membrane fragments from the liver extract which might cosediment with (and thus contaminate) the red cell or ghost pellets. Control incubations of extracts lacking cells or ghosts demonstrated that a negligible quantity of the liver extract ^{32}P-phospholipid should have cosedimented with ghosts under these conditions.

We found that several hours of incubation were required for maximal exchange (see

below). However, we usually restricted the exchange period to 5–6 hr because of the deterioration of the cells and membranes upon prolonged incubation at 37°C with liver extract. Under our incubation conditions, red cell breakage was always well under 1%, as measured by the release of hemoglobin into the supernatant.

Time Course of Exchange

When rat red cells or ghosts were incubated with ^{32}P-labeled liver extract, the membrane fraction increased in specific activity while the supernatant fraction declined (Fig. 1). We interpret this process as phospholipid exchange between the tiny vesicles in the liver extract and the erythrocyte membranes. Within 5 hr, the unsealed ghosts came to the same specific radioactivity as the supernatant, precisely that of the unfractionated incubation mixture. We interpret this as exchange equilibrium. These data suggest that the great preponderance of phospholipids in both the ghosts and the liver extract were accessible and capable of exchange.

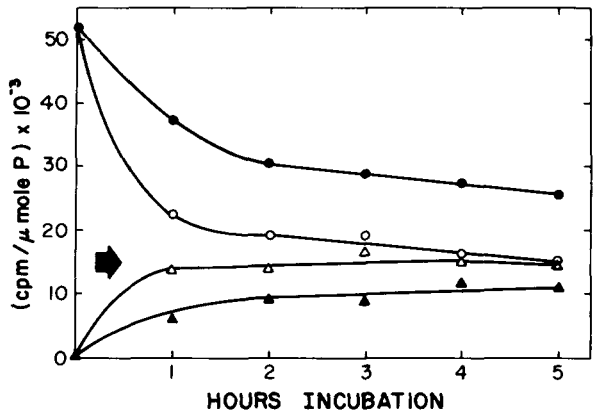

Fig. 1. Time course of exchange of intact rat erythrocytes (closed symbols) and unsealed ghosts (open symbols) with ^{32}P-labeled liver extract phospholipids. After incubation for 0–5 hr the specific radioactivity of the supernatant (circles) and pellet fractions (triangles) was measured (as in Methods). The arrow designates the specific activity of the unfractionated ghost incubation mixture.

In contrast, the specific activity of intact rat erythrocytes and coincubated liver extracts leveled off by 5 hr without reaching equal specific radioactivity. We found in other experiments that complete exchange was not approached even after 18 hr of incubation.

It appears that only a fraction of the phospholipids in intact cells is exchangeable. If so, the size of that fraction can be estimated as follows. The specific activity of the supernatant after prolonged incubation ($\geqslant$ 5 hr in this case) is taken as being equal to the specific activity of the intact red cell phospholipid pool with which it has completely equilibrated. The specific activity of the unexchanged pool is, of course, zero. It can be shown that the fraction (Q) of total membrane phospholipids which exchanged to equilibrium is given by the ratio of the specific radioactivity of the membranes to that of the supernatant phospholipids. For Fig. 1, this quotient is 0.44; i.e., a pool of 44% of the phospholipids in the intact red cell membranes exchanged to equilibrium. The similarity of this value to that predicted for one side of a simple bilayer (0.50) lends support to the premise

that the outer surface phospholipids are completely exchanged while the cytoplasmic surface phospholipids are not exchanged at all.

In perhaps half of the experiments, ghosts did not exchange to completion within 5–6 hr. In 2 experiments similar to that shown in Fig. 1, we found that isotopic exchange in ghosts was only 48% and 89% complete after 6 hr. However, overnight incubation brought about complete exchange between the ghosts and liver extract. It is conceivable that the holes in these unsealed ghosts were sufficiently small to hinder the accessibility of the cytoplasmic surface to the exogenous liver extract exchange system. This effect may have varied from experiment to experiment, resulting in differences in the rate and extent of inner membrane surface exchange. The corresponding intact erythrocyte samples underwent 25% and 36% exchange in 6 hr, which was not substantially increased by a further overnight incubation. Thus, the quotient (Q) defined above clearly represents a minimum value for the fraction of accessible phospholipid when the exchange process has not reached complete equilibrium. An unequal specific activity among the various ^{32}P-phospholipid classes could also confound the interpretation of the exchange quotient, Q.

Figure 2 shows the time course for exchange of phospholipids between ^{32}P-labeled liver extract and human erythrocytes and unsealed ghosts. The results agree well with those obtained using rat red cells (Fig. 1); 95% of ghost phospholipid but only 37% of intact red cell phospholipids were exchanged in the 5 hr incubation.

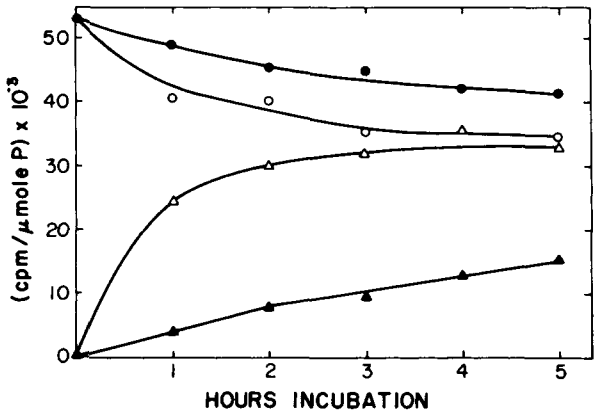

Fig. 2. Time course of exchange of intact human erythrocytes (closed symbols) and unsealed ghosts (open symbols) with ^{32}P-labeled liver extract. After incubation for 0–5 hr, the specific radioactivity of the supernatant (circles) and pellet fractions (triangles) was measured (as in Methods).

Exchange of Individual Phospholipid Classes Between ^{32}P-labeled Liver Extracts and Rat Erythrocyte Membranes

^{32}P-labeled rat liver extracts were incubated for 5 hr with unlabeled rat red cells or ghosts. The specific activity of the major phospholipid classes was determined in the supernatant and pellet fractions. A representative experiment is presented in Table I, A. All four major phospholipids were well labeled, albeit unequally, in the input liver extract (line 1). Line 2 indicates the overall specific activity of phospholipids in the unfractionated mixture of liver extract and ghost membranes; these are the values predicted for the

TABLE I. Exchange of ^{32}P-Labeled Liver Extract Phospholipids with Rat Red Cells and Ghosts*

	Experiment	SM	PC	PE	PS
A	1. Liver extract	9,157	20,172	24,333	10,326
	2. Liver extract + ghosts	2,912	8,775	4,351	4,351
	3. RBC pellet	1,116	5,218	1,860	2,052
	4. RBC supernatant	5,550	7,710	5,345	6,109
	5. Ghost pellet	1,990	8,853	4,665	4,474
	6. Ghost supernatant	4,479	7,343	5,226	3,942
	7. Q(RBC) (line 3/line 4)	0.20	0.68	0.35	0.34
	8. Q(Ghosts) (line 5/line 6)	0.444	1.21	0.89	1.14
	9. % Total RBC phospholipid exchanged	2.6	32.3	7.5	3.7
B	7. Q(RBC)	0.37	0.68	0.33	0.29
	8. Q(Ghosts)	0.31	1.18	0.82	0.97
	9. % Total RBC phospholipid exchanged	4.7	32.3	7.1	3.1
C	7. Q(RBC)	0.045	0.34	0.082	0.16
	8. Q(Ghosts)	0.40	0.88	0.93	0.50
	9. % Total RBC phospholipid exchanged	0.6	16.3	1.8	1.7
D	7. Q(RBC)	0.042	0.28	0.18	0.084
	8. Q(Ghosts)	0.28	0.77	0.52	0.65
	9. % Total RBC phospholipid	0.5	13.4	3.9	0.9

*All values are cpm/μmole lipid P, except lines 7–9. See Text for details.

supernatant and pellet fractions after complete exchange equilibrium. Following incubation, every class of phospholipid in the intact red cell pellet acquired appreciable radioactivity (line 3), while the phospholipids in the liver extract declined in specific radioactivity (line 4). However, no phospholipid class approached equilibrium. In contrast, lines 5 and 6 in Table I, A indicate that the unsealed ghost phospholipids (with the exception of sphingomyelin) approximated complete equilibrium with the liver extract.

The extent to which each membrane phospholipid progressed toward complete equilibration was calculated as the quotient (Q) defined in the previous section for unfractionated lipids (lines 7 and 8). Intact cells showed a limited degree of exchange for all phospholipids. In unsealed ghosts, essentially complete exchange of PC, PE, and PS, occurred, while SM was only partially exchanged. (That the equilibrium specific activity of ghost PC and PS slightly exceeded that found in the supernatant with which they were exchanged is probably experimental error. However, this effect could occur if a certain pool of liver extract phospholipids were both relatively underlabeled and incompletely exchanged.)

The total fraction of phospholipids exchanged by the intact erythrocytes may be calculated by multiplying the fraction of each class exchanged (line 7 of Table I,A) by the respective contribution of each phospholipid to the total red cell pool (given in Ref. 19). These values (line 9 of Table I,A) suggest that a total of 46.1% of the phospholipid was exchanged, predominantly phosphatidylcholine.

Results from three similar experiments are summarized in Table I,B, C, and D to indicate the range of behavior found in this system. Experiment B closely resembled experiment A, except that the exchange of sphingomyelin was as extensive in the intact cells

as in unsealed ghosts. A total of 47.2% of the phospholipid was exchanged (sum of entries in line B.9). In contrast, experiments C and D show more limited exchange of all four phospholipids both in the intact cells and (to a lesser extent) in the ghosts. Only about 20% of the total phospholipids appeared to be exchanged by the intact cells in the latter two experiments.

Exchange of Individual Phospholipid Classes Between ^{32}P-liver Extracts and Human Erythrocyte Membranes

Experiments similar to those shown in Table I were carried out with outdated and fresh human erythrocyte membranes. Table II shows the results of one experiment and summarizes the outcome of four others. In essence, all four phospholipids in human red cells and ghosts showed exchange with the labeled rat liver extract. However, the extent of exchange was significantly less than that found with rat red cell membranes, with intact cells exchanging only about 5–50% as well as unsealed ghosts.

TABLE II. Exchange of ^{32}P-Liver Extract Phospholipids with Human Red Cells and Ghosts*

	Experiment	SM	PC	PE	PS
A	1. Liver extract	24,519	42,656	37,030	17,945
	2. RBC pellet	982	4,808	1,230	1,829
	3. RBC supernatant	16,051	19,402	28,671	12,226
	4. Ghost pellet	2,862	9,081	3,684	4,324
	5. Ghost supernatant	10,392	18,060	10,369	7,342
	6. Q(RBC) (line 2/line 3)	0.061	0.248	0.043	0.150
	7. Q(Ghosts) (line 4/line 5)	0.275	0.503	0.355	0.589
	8. % Total RBC phospholipid exchanged	1.7	7.5	1.2	2.0
B	6. Q(RBC)	0.0373	0.312	0.0615	0.0906
	7. Q(Ghosts)	0.148	0.914	0.808	0.957
	8. % Total RBC phospholipid exchanged	1.04	9.4	1.7	1.2
C	6. Q(RBC)	0.0305	0.3505	0.074	0.264
	7. Q(Ghosts)	0.153	0.742	0.6155	0.742
	8. % Total RBC phospholipid exchanged	0.85	10.6	2.1	3.6
D	6. Q(RBC)	0.0588	0.3586	0.1119	0.0998
	7. Q(Ghosts)	0.1557	0.670	0.481	0.7273
	8. % Total RBC phospholipid exchanged	1.65	10.8	3.2	1.3
E	6. Q(RBC)	0.0150	0.1726	0.0678	0.0836
	7. Q(Ghosts)	0.1850	0.941	0.9375	1.5795
	8. % Total RBC phospholipid exchanged	0.42	5.2	1.9	1.1

*All values are cpm/μmoles P, except lines 6–8. See Text for details.

Exchange of Individual Phospholipid Classes Between ^{32}P-labeled Rat Erythrocyte Membranes and Unlabeled Rat Liver Extracts

To verify the above results, a converse experiment was performed wherein labeled red blood cell membranes, obtained from rats administered $Na_2H^{32}PO_4$ the night before,

were incubated with unlabeled liver extract. Labeled phospholipids could have been incorporated into the erythrocyte membranes either during erythropoesis or by an exchange between circulating red cells and plasma lipoproteins. Because the labeling period is short relative to the duration of erythrocyte maturation in the marrow and because only a few percent of the circulating rat erythrocyte pool is renewed each day, the fairly rapid process of exchange between red cell and plasma phospholipids (12) probably accounts for most of the erythrocyte labeling. If so, a large fraction of the radioactivity might be confined to the outer lamella of these membranes.

The time course of the phospholipid exchange between ^{32}P-labeled erythrocytes and ghosts and unlabeled liver extract is represented in Fig. 3. The liver extract increased in its specific radioactivity at the expense of the membranes in incubations with both in-

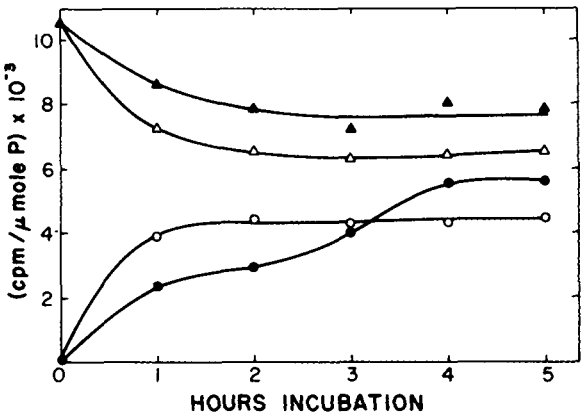

Fig. 3. Time course of exchange of ^{32}P-labeled rat erythrocytes (closed symbols) and ^{32}P-labeled unsealed ghosts (open symbols) with liver extracts. After incubation for 0–5 hr, the specific radioactivity of the supernatant (circles) and pellet fractions (triangles) was measured (as in Methods).

tact cells and unsealed ghosts. Control experiments have indicated that the release of ^{32}P-phospholipids to the supernatant medium does not occur in the absence of the liver extract. It is noteworthy that the specific activity of the supernatant fraction in the intact cell experiment exceeded that of the ghosts both in Fig. 3 and in a confirmatory experiment. This phenomenon was not seen when unlabeled cells were equilibrated with ^{32}P-labeled liver extract. This effect could be explained if the erythrocyte membranes acquired most of their label in vivo by an exchange reaction with the plasma as discussed above, since the phospholipid pool available for exchange would be of higher specific activity in intact cell membranes than ghosts. These complexities discouraged attempts at estimating the fraction of total phospholipid exchanged.

Table III demonstrates that all four of the major phospholipid classes in these red cell membranes were labeled and were capable of exchange with unlabeled liver extract. As in Fig. 3, the supernatant fractions incubated with intact red cells reached a higher specific activity than those incubated with ghosts (except for PC). This experiment confirms the premise that all four phospholipids are accessible for exchange in intact red cells.

TABLE III. Exchange of ^{32}P-Labeled Rat Cell and Ghost Phospholipids with Liver Extracts*

	SM	PC	PE	PS
1. Input membranes	8,700	13,133	3,201	3,340
2. RBC pellet	8,540	11,063	2,956	2,373
3. RBC supernatant	4,368	7,852	2,585	2,252
4. Ghost pellet	7,767	10,670	2,956	2,484
5. Ghost supernatant	4,124	8,348	2,077	2,006

*All values are cpm/μmole lipid P. See Text for details.

DISCUSSION

Four principal inferences may be drawn from this study which bear comment.

1. A phospholipid exchange reaction is being observed between the liver extract and the erythrocyte membrane. It has been well established that the rat liver cytoplasm contains proteins which specifically catalyze the exchange of various phospholipids between microsomal vesicles, mitochondria, and a variety of other membranes and lipid substrates (cf. Refs. 11–13). Rothman and Dawidowicz (20) recently reported that a purified calf liver protein stimulated the exchange of PC between unsealed human ghosts and liposomes. However, intact erythrocytes were not an active substrate in that system (20,21). In the present study, all four major phospholipids were transferred between a crude liver extract and both intact red cells and ghosts. This difference between these results and those just cited may reflect species specificity, since Tables I and II indicate that exchange is catalyzed more effectively with homologous red cells as a substrate than human cells. Furthermore, we used a long incubation period ($\geqslant$ 5 hr) compared to the aforementioned studies (20,21). Finally, there may be extra components in the crude liver extract which facilitate the exchange reaction with intact erythrocytes, just as sphingomyelinase treatment stimulated the exchange of PC in intact cells (21).

Could it be that cross-contamination of the supernatant (liver extract) and pellet (erythrocyte membrane) fractions contributes to the observed changes in their specific radioactivities? Control studies have shown that intact red cells do not release significant phospholipids to media lacking the liver extract. However, following incubation at 37°C, some ^{32}P-phospholipids can be pelleted from rat liver extracts (minus erythrocyte membranes) at low centrifugal forces, presumably because of the aggregation of small membrane vesicles. This effect cannot account for our results, since (a) the amount of ^{32}P-lipid pelleted is small in the absence of erythrocyte membranes; (b) the specific activity in the supernatant fraction should not be altered by this effect; and (c) this mechanism would not account for the unequal exchange rates observed among the four major phospholipids. Evidence for true exchange is also afforded by the reciprocal changes in the specific activity of ^{32}P-labeled erythrocyte membranes and coincubated, unlabeled liver extract. Since bulk transfer of liver extract lipids to the pellet could, nevertheless, occur by aggregation or fusion with the erythrocyte membranes, the decline in the specific activity in the supernatant may be a more reliable indicator of phospholipid exchange than the increase in the specific activity of the pellet, particularly when only small amounts of label are transferred.

2. All of the major phospholipid molecules are accessible for exchange between ghosts and liver extracts. Experiments in which the supernatant and pellet (ghost) fractions reach essentially the same specific activity (such as those shown in Figs. 1 and 2 and Tables I and II) indicate that no significant pool of phospholipids in either fraction is unexchangeable. However, unequal and incomplete exchange of various phospholipid classes is frequently observed. Factors which might limit rate of exchange include (a) unequal activities of the specific exchange factors for the various phospholipids; (b) the unequal abundance and accessibility of given phospholipid classes in both the donor and acceptor membranes; (c) differences among phospholipids in their binding to membrane proteins, their fatty acyl chain composition, etc.

3. The phospholipids at the inner (cytoplasmic) surface of the intact erythrocyte membrane lipid bilayer do not exchange with the exogenous liver extract. Since the extent of phospholipid exchange in intact cells is no more than half that found in ghosts, a plausible inference is that the phospholipids of the inner half of the membrane are not accessible to the external medium, even over 18 hr of incubation. This is also the conclusion drawn from labeling and phospholipase digestion studies (see Introduction). No evidence of transverse diffusion of phospholipids across the plane of the red cell membrane is afforded by any of these studies or by the demonstration of glycolipid asymmetry in this membrane (2). Furthermore, it appears that the purified PC exchange protein was unable to interact with the inner lamella of PC liposomes while fully exchanging the outer monolayer (20).

However, it is also conceivable that the rat liver extract employed here contains factors capable of exchanging with domains of phospholipid at both surfaces. Incomplete exchange might then be a result of the unreactivity of certain populations of phospholipids at each red cell membrane surface (for example, protein-bound lipids). Purified exchange factors studied to date need not share this property.

4. All four major phospholipid classes in intact rat and human red cells are partially accessible to the liver exchange factors. This premise accounts for the decline in the specific activity of all four phospholipid classes in ^{32}P-labeled liver extracts after incubation with intact red cells (Tables I and II) and for the increasing labeling of these components in liver extracts incubated with ^{32}P-labeled rat erythrocytes (Table III).

However, a possible effect of phosphatidyl transferases must also be mentioned. Rat liver microsomal phosphatidyl transferases have been reported to introduce free L-serine, choline, and ethanolamine into microsomal phospholipids by an exchange of bases (22,23). It is thus conceivable that only PC was exchanged between intact red cells and liver microsomes and that this phospholipid was subsequently converted to other phosphatide species by base exchange. This mechanism is probably not contributory here, however, since the substantial levels of both free bases and divalent cations required for the reaction in vitro were not available in our system. Furthermore, these enzymes are confined to the microsomes and would presumably not be able to alter the phospholipid profile of the red cell membranes. (These considerations, however, indicate the caution with which data from poorly characterized systems such as rat liver extracts must be interpreted and point to the utility of purified exchange factors and lipid dispersions.)

Phosphatidyl choline was the most reactive phospholipid in our system; up to two-thirds was exchanged between intact cells and the liver extract. Studies using phospholipases as probes have similarly shown that 55–76% of PC in intact erythrocytes was

hydrolyzed (6–8). Furthermore, Reed (24) found 60% of intact red cell PC exchangeable with plasma.

In the case of phosphatidylethanolamine, Bretscher (4) reported that ghosts are labeled up to 50 times more readily than intact cells by formylmethionylsulphone methyl phosphate. However, Gordesky and Marinetti (5) found that up to 33% of intact human red cell PE reacted with trinitrobenzenesulfonate. Zwaal et al. (6) reported that bee venom phospholipase A_2 hydrolyzed 9% of the PE of intact human erythrocytes; Verkleij et al. (8) showed that this fraction increases to 20% if sphingomyelinase is present. Our data suggest that PE is exchanged in intact rat and, particularly, human red cells about 10–30% as well as in ghosts. These values fall within the cited range obtained with other probes.

In the case of phosphatidyl serine, a difference exists between our conclusions and those of others. Essentially no PS was covalently labeled (4,5) or digested (6–8) in intact human red cells, whereas this phospholipid was reactive in unsealed ghosts, as well as in inside-out vesicles (9). In contrast, our data (Tables I–III) indicate that PS in intact rat and human erythrocytes does exchange with liver extracts, although perhaps only 5–35% as well as in ghosts.

Could the negative results in the aforementioned probe experiments be caused by greater unreactivity of PS at the outer membrane surface of native intact cells as compared to the isolated membranes? Papahadjopoulos and Weiss (25), for example, have shown that the reactivity to covalent ligands of both PE and PS in phospholipid vesicles was strongly dependent on their physical state. Phosphatidyl serine is particularly unreactive. Likewise, human erythrocytes were shown to be quite resistant to certain purified phospholipases (6,8,26) or to PC exchange (21) until their membranes were altered by sublytic perturbation. It is conceivable, therefore, that there is PE and PS at the outer surface of erythrocytes which resists chemical and enzymic attack but is capable of exchange with rat liver extracts. The alternative hypothesis – that all PE and PS molecules are normally confined to the cytoplasmic surface (4) yet can be exchanged across the membrane – cannot be ruled out. However, if the inner surface phospholipids were accessible for such exchange, why do intact red cell preparations exchange no more than half of their phospholipid complement? These questions cannot be resolved at present but frame the context of future studies.

Finally, it is relevant to note that (for man and dog) PC and SM exchange well between erythrocytes and plasma, while little PS and PE exchange can be detected (24). We have made similar observations on rats in vivo (A.R. Tarlov and M.S. Gibson, unpublished). However, human and rat plasmas are nearly devoid of PE and PS (19). One could speculate that the distribution of phospholipids in newly assembled erythrocyte membranes is more nearly symmetrical but that remodeling gradually occurs by nonuniform phospholipid exchange with the PE- and PS-poor plasma during the lifetime of the circulating red cell. Exchangeable red cell PE and PS might then be richest in the youngest red cells and depleted in the oldest. Residual outer-surface PE and PS, furthermore, could be a firmly bound fraction resistant not only to depletion in the circulation but also poorly reactive with certain probes in vitro.

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