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Concomitant Alterations of Sodium Flux and Membrane Phospholipid Metabolism in Red Blood Cells: Studies in Hereditary Spherocytosis *

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Summary. **The role of membrane phosphatides in transport processes has been investigated in red cells from splenectomized patients with hereditary spherocytosis (HS).**

Incorporation of inorganic ^{32}phosphate into the membrane phosphatides of HS red cells was approximately twice normal, coinciding with the nearly two-fold increment in flux of sodium ions in the cells.

A consistent, inordinate increase in specific activity of a chromatographic fraction containing phosphatidylserine provided the bulk of the over-all increase in labeling of HS red cell phosphatides. The specific activity of phosphatidic acid was increased but not consistently.

Radioactivity of the "acidic phosphatides" (phosphatidylserine and phosphatidic acid fractions) decreased, in general, when the sodium flux was low, i.e., when the cells were suspended in media of low sodium content. When the cation flux was elevated (hypotonic media), there was a marked (*ca.* 35%) increase in the labeling of phosphatidylserine fractions. Normal red cells whose permeability to cations was increased by exposure to 0.5 N butanol also exhibited increased labeling of acidic phosphatides.

Considerations of the stoichiometry of cation transport and phosphatide labeling make it unlikely that phospholipids act directly as carrier molecules for cations in red cell membranes. On the other hand, the involvement of these lipid substances in cation movements is substantiated by correlating several different states of sodium flux with the labeling of the phosphatidic acid and phosphatidylserine fractions.

Introduction

Phospholipids have been implicated in cation and other transport systems by the findings of numerous investigators (1–7). The possibility that these compounds might play a central role in transport phenomena stems partly from the facts that they are important constituents of cellular membranes and that certain members of the class have considerable ability to bind cations (8–10). The phosphatides that bear a net negative charge at physiologic pH levels ("acidic" phosphatides such as phosphatidic acid, phosphatidylserine, and inositol phosphatides) are especially active in the latter regard, and the extensive work of the Hokins (11) has demonstrated that the metabolism of phosphatidic acid and phosphatidylinositol is most markedly affected during transport processes. For example, when sodium transport in the avian nasal gland is stimulated by the action of acetyl-

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choline, the Hokins have found that the metabolism of phosphatidic acid is greatly enhanced (11). These observations, and others, led to the postulation some years ago of a direct role of phosphatidic acid as a "carrier" of the sodium ion, involving its dephosphorylation by a phosphatase and re-formation from diglyceride by a specific kinase and ATP (12, 13). If phosphatidic acid were a carrier of sodium ions, such a cycle would have the over-all effect of linking sodium transport and ATP utilization. Stimulation of sodium transport would be expected to result in increased turnover of the phosphate moiety of phosphatidic acid. However, when the kinetics of cation transport and phosphatidic acid turnover are examined in various tissues, serious discrepancies are evident (14). Furthermore, it has been shown that three sodium ions are transported per ATP molecule turned over (15, 16), whereas, if phosphatidic acid were indeed the carrier, the maximal number of sodium ions transported per ATP would be two. These discrepancies and the fact that incorporation of radioactive phosphate into other acidic phosphatides such as phosphatidylinositol has also been found to respond to alterations in sodium transport (17) have necessitated the formulation of other ideas concerning the role of complex phosphatides in cation transport (11). Finally, the altered phosphatide metabolism evoked by pharmacologic or physical stimulation of tissues *in vitro* may not be linked specifically to changes in cation transport but instead may reflect other unrelated phenomena induced by the stimulating agent.

In the present studies, some aspects of membrane-lipid metabolism in red cells from patients with hereditary spherocytosis (HS) have been investigated by examining the incorporation of inorganic ^{32}phosphate ($^{32}P_i$) into various phosphatide species under different experimental conditions. The membranes of HS red cells leak sodium ions at inordinate rates (18, 19). Under optimal conditions, as in the general circulation, HS red cells maintain normal cation distributions and thus preserve their viability by nearly doubling their active pumping of sodium outward against the cation gradient (19, 20). Failure of this accelerated uphill transport results in accumulation of intracellular sodium and water, followed by osmotic swelling and ultimately hemolysis (19). The survival of spherocytes becomes normal after splenectomy (21), even though they continue to manifest almost double the normal flux of sodium (19). These cells, therefore, offer a unique opportunity to investigate the possible relationship of membrane phospholipid metabolism and sodium movements in red cells of normal mean age. A minimum of experimental manipulation of the cells is involved in such studies, and conditions of increased flux of sodium are maintained.

The results obtained with HS cells in this study have been strengthened by the following experimental maneuvers: Sodium movements were *a*) diminished and *b*) increased in both normal and HS red cells. Further, *c*) the intracellular levels of sodium were raised acutely, and *d*) transport of cations was inhibited by ouabain. In all cases, the incorporation of $^{32}P_i$ into phosphatides was determined. The results obtained provide support for the idea that phospholipids are involved in cation flux and also suggest mechanisms for the hemolytic process in HS itself. These results have been partially presented in preliminary form elsewhere (22).

Methods

Incubation procedure. Five patients, all of whom had been splenectomized in the past, served as donors of HS red cells. The patients, from four families, demonstrated normal routine blood counts (23), including reticulocyte percentages, at the time of the studies. The following characteristics, considered typical of the disease (24), were present in every donor: *a*) A congenital hemolytic anemia affected at least two family members; *b*) complete clinical remission occurred after splenectomy; *c*) spherocytes were demonstrable on peripheral smear, and associated with this, increased osmotic fragilities of fresh and incubated blood were noted. Finally, *d*) an abnormal degree of autohemolysis occurred after prolonged incubation; this was partially corrected by adding glucose. These five donors have been utilized in previous studies of the pathogenesis of HS (19, 25). Normal cells were obtained from healthy male volunteers.

Fresh blood drawn into solution [1] was centrifuged and the buffy coat removed, and the cells were resuspended in the incubation medium. After recentrifugation and a second removal of any remaining buffy coat, the red cells were resuspended in the incubation medium to give a hematocrit of 50%. Leukocytes in these cell suspensions numbered less than 1,000 and platelets less than

[1] Abbott Laboratories, North Chicago, Ill. Each 10 ml contains dextrose, 132 mg; sodium citrate, 250 mg; and citric acid, 80 mg.

TABLE I

*Incorporation of inorganic phosphate ($^{32}P_i$) into the phospholipids of red blood cells (RBC)**

Experiment	Normal			Hereditary spherocytosis (HS)			Ratio of SA of HS/normal
	Phospholipid	Radioactivity	SA	Phospholipid	Radioactivity	SA	
	μg P/ml RBC	*cpm/ml RBC*	*cpm/μg P*	*μg P/ml RBC*	*cpm/ml RBC*	*cpm/μg P*	
1	108	34,800	322	107	102,000	953	2.96
2	101	29,600	292	100	65,100	651	2.23
3	100	25,000	250	88	49,800	567	2.26
4	112	94,500	844	134	162,000	1,210	1.44
5	96	191,000	1,990	106	393,000	3,710	1.86
6	102	220,000	2,160	106	376,000	3,550	1.65
Mean ± SE	103 ± 2			107 ± 6			2.07† ±0.23

* Washed red cell suspensions of the same red cell concentration from four normal and five HS patients were incubated in parallel in identical media for 4 hours at 37° C with $^{32}P_i$ (approximately 100 μc per ml). The phosphate concentration of the suspending media varied as follows: experiments 1 to 3, 30 mmoles per L; experiment 4, 12 mmoles per L; experiments 5 and 6, 6 mmoles per L.

† p value for difference between HS and normal cells < 0.005.

20,000 per mm^3. The incubation medium, described previously (19), was, unless otherwise stated, a buffered 5% dialyzed human serum albumin [2] solution containing the following constituents: K^+, 3.5 mmoles per L; Na^+, 160 mmoles per L; $PO_4^=$, 30 mmoles per L; HCO_3^-, 25 mmoles per L; and Cl^-, balance of anions. Glucose was added after dialysis to a final concentration of 22 mmoles per L. The medium was isosmolal with normal plasma as determined by freezing point depression.

The cell suspension was equilibrated to a pH of 7.4 with a 95% oxygen and 5% carbon dioxide mixture before incubation. After removal of a sample of cells for counting of blood elements and hematocrit determination, the remaining cell suspension was divided into 5-ml aliquots. To these was added carrier-free [3] $^{32}P_i$ (approximately 100 μc per ml red cells), and the stoppered flasks were incubated with shaking at 37° C. During the incubation the pH of the system dropped by 0.1 to 0.2 U (cf. 19).

At various intervals, the cell suspensions were removed from the incubation flasks, and the incorporation of label into cellular phospholipids was determined. In all experiments, the phospholipid metabolism of HS red cells was compared to that of red cells from normal subjects incubated at the same time under identical conditions.

[2] Normal human serum albumin was obtained from the Massachusetts Public Health Biologic Laboratories, as a 25% solution, through the courtesy of Dr. R. Pennell. After dialysis against appropriate buffer, the solution was diluted to a final concentration of 5% in buffer. This medium was used rather than plasma because previous studies (26, 27) indicated that many of the phospholipids present in plasma are rapidly (but not predictably) exchangeable with those of the membranes of red cells incubated therein.

[3] Available from IsoServe, Cambridge, Mass., as $H_3{}^{32}PO_4$ in 0.1 N HCl; the solution was adjusted to neutral pH with 0.5 N NaOH.

Extraction and analysis of red cell lipids. After incubation, the red cell suspensions were washed three times with large volumes of ice cold phosphate-buffered isotonic saline solution (final PO_4 concentration, 40 mmoles per L) and again made to original volume. Lipids were extracted from the cell suspension by procedure III of Ways and Hanahan (28). Although this procedure has been shown to yield lipid virtually free of nonlipid contaminants, it was modified in a minor way by increasing the number of aqueous washes of the final lipid extract from one to three. This assured even more fully the absence of contamination of lipid by inorganic phosphate and other water-soluble phosphorylated compounds. To reduce the heme-catalyzed oxidation of lipids (29), we performed the initial extraction steps in an atmosphere of dry nitrogen.[4] Samples of lipid extract were analyzed for phosphorus by the method of Lowry and co-workers (30) scaled up to a convenient level and were counted after drying on aluminum planchets in a Nuclear Chicago gas flow counter (model 186A). Duplicate planchets were counted under such conditions as to maintain a counting error no greater than 1%. Agreement between duplicate samples was within ±2%. The remaining lipid extract in small volumes of chloroform-methanol (2:1) was spotted on specially cut, long, narrow (2- × 16-inch) glass plates coated with silica gel G as described by Stahl (31), and ascending thin layer chromatographic separation of the individual phosphatides was performed with the solvent system, chloroform : methanol : acetic acid : water (500 : 300 : 80 : 40, vol : vol) as described by Skipski, Peterson, and Barclay (32). The separated phosphatides were localized with iodine or sulfuric acid spray and were identified by comparison with reference compounds,[5] by group-specific sprays such as Ninhydrin

[4] Good results were obtained by extracting under nitrogen in glove boxes available from Instruments for Research, Cheltenham, Pa.

[5] The authors are grateful to Dr. E. P. Kennedy, who generously provided phosphatidylinositol-^{3}H and phos-

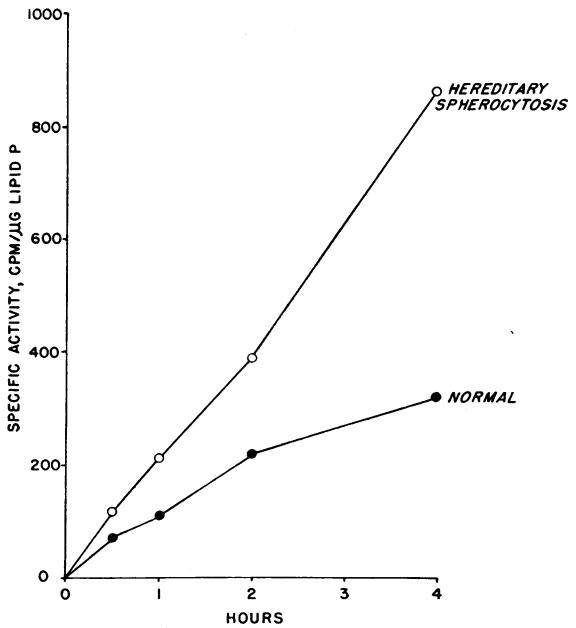

FIG. 1. THE INCORPORATION OF INORGANIC PHOSPHATE ($^{32}P_i$) INTO THE PHOSPHATIDES OF HEREDITARY SPHEROCYTOSIS (HS) AND NORMAL RED CELLS. When incubated at 37° C in identical media with $^{32}P_i$, the phosphatides of washed HS red cells incorporated more label than did those of normal cells at all time intervals. The study depicted is representative of six performed utilizing red cells from five HS and four normal donors.

for α-amino groups and the Dragendorff reagent for choline, and by deacylation and chromatography of water-soluble products on paper with the phenol:ethanol: acetic acid (100:12:10) solvent system described by Dawson, Hemington, and Davenport (33).

Thin layer chromatography on long glass plates produced excellent separation of the original phosphatides and permitted automated scanning of the developed plate for radioactivity on a Baird Atomic scanner model RSC 363. After development, scanning, and spraying, the area of silica gel containing each phosphatide was removed from the plate, and a weighed sample was digested and analyzed for phosphorus by modifications of the methods of Lowry and co-workers (30) and Berenblum and Chain (34) as follows: Digestion was performed in perchloric acid and color developed according to Lowry and co-workers (30). After its development, the blue phosphomolybdous complex was readily extracted into 4 ml of isobutanol (34) and the optical density determined at 790 mμ on a sample after centrifugation.[6] A sample of the isobutanol solution was transferred to aluminum planchets for counting of radioactivity as described above. From these data specific activities (counts per minute per microgram phosphorus) of the individual phosphatides could be calculated.

phatidylserine-^{14}C, and to Dr. John Law for providing phosphatidic acid and phosphatidylethanolamine for use as standard reference compounds.

[6] This method of dealing with thin layer chromatography fractions was kindly made available to us by Dr. A. W. Shafer.

Results

The uptake of $^{32}P_i$ into the phospholipids of HS and normal red blood cells.

As shown in Table I the total quantity of phospholipid that was extractable from HS red cells did not differ in any consistent fashion from that of normal cells. On the other hand, the incorporation of label into phospholipid from added $^{32}P_i$ was significantly greater in HS red cells than in normal cells in all six experiments. After 4 hours of incubation, the specific activity of HS red cell phospholipid averaged slightly more than twice that in the normal red cells, which had been incubated simultaneously under identical conditions. The numbers of leukocytes and platelets in the cell suspensions in these experiments varied randomly and did not correlate with the results shown.

An increased rate of incorporation of $^{32}P_i$ into HS red cell phospholipid was manifest at all intervals studied, the shortest being 30 minutes, as shown in Figure 1. This increased labeling of lipids was not associated with an abnormally rapid influx of inorganic phosphate itself into HS cells; the over-all uptake of radioactivity ($^{32}P_i$) from the incubation media was determined to be no different in HS cells from that in normal red cell suspensions, in confirmation of previous results of others (35).

The labeling of individual phosphatides in HS and normal red cells

The lower portion of Figure 2 demonstrates the silica gel chromatographic separation of red cell phosphatides on long glass plates. The material present in each numbered zone was identified as follows:

Zone 1 contains material remaining at the origin after chromatographic development, including contaminating inorganic phosphate or other water-soluble, nonlipid phosphorylated compounds. Polyphosphoinositides may also be present in this zone (36).

Zone 2 material was identified as sphingomyelin by agreement with R_f values reported by Skipski

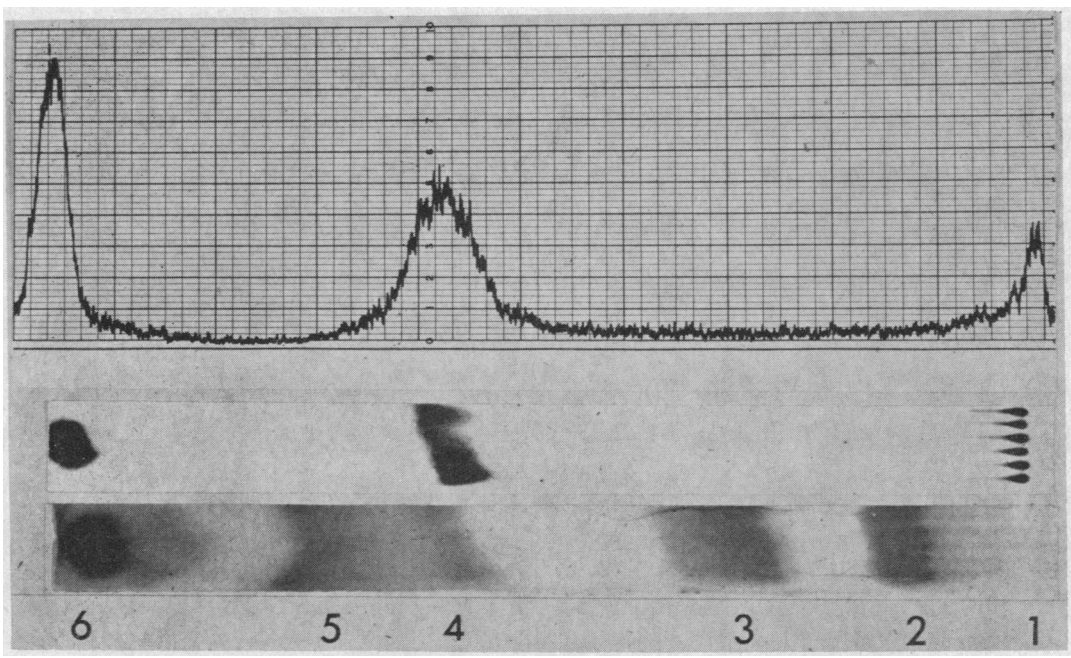

FIG. 2. THIN LAYER CHROMATOGRAPHY OF ^{32}P-LABELED PHOSPHATIDES FROM RED CELLS. The extracted phosphatides from red cells incubated with $^{32}P_i$ are chromatographically separated into six zones on 2- × 16-inch glass plates coated with silica gel. The zones are revealed by spraying with H_2SO_4 and heating (bottom). For identification of individual phosphatides, see text. By radioautography (middle), origin material (zone 1), phosphatidylserine (zone 4), and phosphatidic acid (zone 6) are heavily labeled. This is also demonstrated in the top portion by automated radioactivity scanning (see text).

and associates (32), by comparison with authentic samples in this laboratory, by its positive reaction for choline when sprayed with the Dragendorff reagent, and by the extent of its occurrence in the total phosphatide pool, which corresponds with previously published (28, 37) values for sphingomyelin in red cells.

Zone 3 was identified as phosphatidylcholine by the kinds of criteria noted for zone 2. It had a chromatographic mobility identical to that of a sample of authentic egg lecithin, in the same system. Deacylation of material in this zone by mild alkaline hydrolysis followed by rechromatography on paper (33) yielded a compound running with the same R_f as glycerophosphorylcholine obtained from standard synthetic lecithin.[7]

Zone 4 was identified as phosphatidylserine by the coincidence of its R_f value with reference standard material under the same chromatographic conditions. The compound is Ninhydrin positive, and after deacylation and rechromatography on paper (33), a Ninhydrin-positive compound was obtained with mobility identical to that of standard glycerophosphorylserine derived from synthetic phosphatidylserine. After prolonged storage at 4° C, spontaneous partial deacylation of the material from red cells yielded a Ninhydrin-positive compound that upon rechromatography on silica gel ran coincidentally with a product (lysophosphatidylserine) formed in the same way from authentic phosphatidylserine. The presence of a very small quantity of phosphatidylinositol in this zone is a possibility that cannot be completely excluded.

Zone 5 was identified as phosphatidylethanolamine by its R_f value, which was identical to that of reference standard material [5] during simultaneous silica gel chromatography. The compound is Ninhydrin positive and yielded a water-soluble Ninhydrin-positive substance with the mobility of standard glycerophosphorylethanolamine derived from phosphatidylethanolamine after mild alkaline hydrolysis and rechromatography on paper (33).

[7] Generously provided by Dr. Erich Baer.

Zone 6 contains neutral lipids and phosphatidic acid, the latter identified by the matching of its R_f with that of reference standard material [5] during silica gel chromatography. Cardiolipin, whose presence in red cells is not yet certain (28, 33, 37), might also be present in this zone.

Radioautography of labeled and chromatographically separated phosphatides from red cells incubated with $^{32}P_i$ for 4 hours is shown in the middle portion of Figure 2 (the corresponding stained plate is shown in the lower portion of the Figure). Origin material (zone 1), phosphatidylserine (and possibly phosphatidylinositol) (zone 4), and phosphatidic acid (zone 6) are labeled. Sphingomyelin (zone 2), lecithin (zone 3), and phosphatidylethanolamine (zone 5), although comprising approximately 85% of the total phospholipid content of red cells (28, 37), had not significantly incorporated $^{32}P_i$ during the time interval of this incubation and under the conditions of the detection techniques employed. The upper portion of Figure 2 shows the radioactive scan of the thin layer plate portrayed in the lower portion. The symmetry of the peaks suggests the homogeneity of radioactive compounds.

Phosphatidylinositol, whose presence in red cells is controversial (28, 33), behaves similarly to phosphatidylserine in the chromatographic system utilized in Figure 2. We attempted, as follows, to determine whether labeled inositide might indeed be present in zone 4: The material from this zone was eluted (32), deacylated by mild alkaline treatment (33), and mixed with standard, nonradioactive glycerophosphorylserine (GPS) and radioactive glycerophosphorylinositol (GPI) tritiated in the inositol moiety.[5] After rechromatography on paper (33), a homogeneous ^{32}P-labeled peak was found to be exactly coincident with the Ninhydrin-positive, standard GPS spot. A homogeneous peak of radioactivity due to tritium alone [8] ran more slowly with an R_f two-thirds that of the peak containing ^{32}P. We tentatively concluded, therefore, that phosphatidylserine is the only labeled compound significantly present in zone 4 of our thin layer chromatograms. In view of the faint element of doubt that remains, however, this zone will be referred to as the "phosphatidylserine fraction."

The over-all proportions of individual phospholipids in HS red cells were found to be the same as in normal cells, confirming the results of others (38, 39).[9] However, the incorporation of $^{32}P_i$ into the various membrane phosphatides of these two cell types was clearly different. As shown in Table II, red cell phosphatidic acid was highly labeled after 4 hours of incubation with $^{32}P_i$. In three of six experiments, the specific activity of this compound was the same in HS and in normal red cells, whereas in the remaining experiments, its activity was higher in spherocytes.

The specific activity of the phosphatidylserine fraction sometimes exceeded that of phosphatidic acid, but more importantly, was consistently in-

[8] Counted as paper strips 1 cm × 2½ cm in a liquid scintillation spectrometer, Packard Instrument Co., La Grange, Ill.

[9] The mean percentage distribution of phosphatides in HS and normal red cells was as follows: phosphatidylcholine (lecithin), 32%; phosphatidylethanolamine, 25%; sphingomyelin, 25%; phosphatidylserine, 10%; phosphatidic acid, 3%; unknown, 5%.

TABLE II

*Incorporation of $^{32}P_i$ into the "acidic" phosphatides of red blood cells**

Experiment	Phosphatidic acid fraction			Phosphatidylserine fraction		
	Normal	HS	HS/normal	Normal	HS	HS/normal
		cpm/μg P			*cpm/μg P*	
1	6,830	7,020	1.02	4,900	7,550	1.54
2	4,270	10,200	2.39	1,900	4,080	2.05
3	3,470	9,390	2.70	1,450	2,220	1.53
4	9,950	9,970	1.00	5,020	13,500	2.69
5	23,200	22,900	0.99	17,000	42,200	2.48
6	10,400	34,300	3.30	16,000	36,200	2.26
		Mean ± SE	1.90 ± 0.42			2.09 ± 0.20
			p = 0.10			p = <0.005

* The conditions of incubation are described in Table I.

creased in HS red cells relative to normal cells (Table II), and this was so at all time intervals studied (Figure 3).

Effect of decreased concentration of sodium on red cell phospholipid metabolism

When red cells are suspended in media of low sodium content, and the passive diffusion of this ion into the cells is thereby diminished, the requirement for active transport of sodium in the opposite direction is reduced. Previous studies have shown that incubation in media in which choline replaces sodium decreases the abnormal osmotic swelling and autohemolysis that occur in incubated HS red cells (19). As shown in Table III, the incorporation of $^{32}P_i$ into phosphatidic acid was reduced by an average of 35% in three experiments in which HS and normal red cells were suspended in media containing choline rather than sodium. The labeling of the phosphatidylserine fraction was also significantly diminished in the one experiment in which HS cells were suspended in choline media; in normal cells this effect was not consistently observed.

Effects of increased flux of cations on red cell phospholipid metabolism

Suspension of red cells in mildly hypotonic medium produces osmotic swelling and increased membrane permeability to a variety of small molecules. In response, active transport of cations by these cells accelerates (40). Previous studies, both in red cells (19) and in brain tissue (41), have indicated that by such treatment an ouabain inhibitable ATPase of the cell membrane is stimulated. As shown in Table IV, the labeling of the phosphatidylserine fraction is accelerated by suspension of red cells in hypotonic media that do not cause hemolysis. In the five experiments performed, the effect was more pronounced in normal red cells (three experiments) than in HS red cells (two experiments) whose cation permeability and phosphatide metabolism are already higher than normal. In these experiments, the incorporation of $^{32}P_i$ into red cell phosphatides other than those of the phosphatidylserine fraction was not altered by hypotonicity in any consistent fashion.

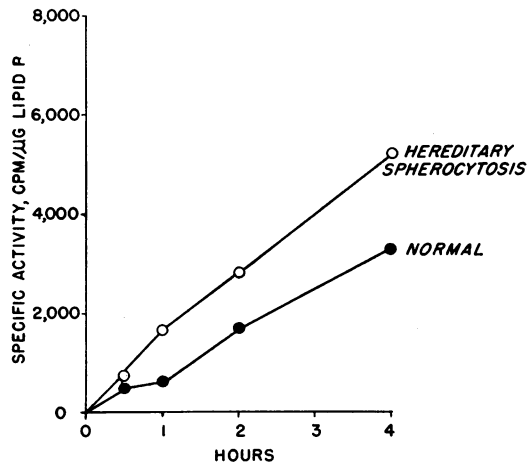

FIG. 3. THE INCORPORATION OF $^{32}P_i$ INTO THE PHOSPHATIDYLSERINE FRACTION OF HS AND NORMAL RED CELLS. The specific activity of this phosphatidylserine fraction from HS red cells exceeds that from normal cells at all time intervals. The conditions of incubation are given in the legend to Figure 1.

TABLE III

*Effect of extracellular sodium on the labeling of red cell phosphatides**

Red cells	Sodium in medium	Phosphatidic acid fraction		Phosphatidylserine fraction	
		cpm/μg P	*% change*	*cpm/μg P*	*% change*
HS	+	22,900		42,200	
	−	18,900	−17	27,900	−34
Normal	+	654		6,750	
	−	390	−40	5,500	−19
Normal	+	23,200		17,000	
	−	12,100	−48	17,700	+4

* Two aliquots of the same red cells were incubated in parallel with $^{32}P_i$ for 4 hours at 37° C in identical suspending media except for sodium content. In sodium-free media, an equivalent amount of choline chloride replaced sodium chloride.

TABLE IV

*Effect of osmotic swelling on labeling of the phosphatidylserine fraction in red cells**

Experiment	Medium: Isotonic (a)	Medium: Hypotonic (b)	% effect $\frac{b-a}{a} \times 100$
	cpm/μg P		
HS 1	13,500	16,800	+24
HS 2	42,200	43,400	+ 3
Normal 1	5,020	8,870	+77
Normal 2	17,000	22,700	+34
Normal 3	1,720	2,370	+38
			35 ± 12†

* Two aliquots of the same red cell suspension were incubated in parallel with $^{32}P_i$ for 4 hours at 37° C in media that differed only in respect to tonicity. Isotonic media were 300, and hypotonic media 200 milliosmolal. HS represents experiments with spherocytes from two donors. Normal red cells were obtained from two donors.

† p value for difference between experiments in isotonic and hypotonic media = 0.03.

For example, in the case of phosphatidic acid, effects due to hypotonicity varied from a decrease of 50% to an increase of 200% in five experiments. The effects with respect to total phosphatide were from − 30% to + 25%.

Effects of Na loading on red cell phospholipid metabolism

A reversible increase in membrane permeability can be produced in red cells by exposing them for brief periods to cold 0.5 N butanol (42, 43). During this period of increased permeability, red cells rapidly incorporate cations from the external medium into their intracellular space. After removal of butanol and washing of the cells, membrane permeability becomes normal, and cells loaded with extracellular cations are thereby obtained (44). The labeling of phospholipids from Na-loaded red cells, which had been prepared by exposure to butanol while suspended in media of high Na^+ concentration, was compared to that of similarly treated "control" cells whose Na content was normal after exposure to butanol in media of low Na (high K) concentration. As shown in Table V, the specific activity of the total phospholipid pool and of the phosphatidylserine fraction was increased by about 30% in Na-loaded red cells relative to control cells with normal Na content. In a further experiment restricted to measurements on total phosphatides, a stimulation of 30% due to Na^+ loading was obtained. Labeling of phosphatidic acid was even more markedly enhanced in the Na-loaded cells.

TABLE V

*Effect of Na loading on labeling of RBC phospholipids**

	"Control" RBC	Na^+-loaded RBC
	cpm/μg P	
Total phospholipid	1,125	1,436
Phosphatidylserine fraction	3,383	4,440
Phosphatidic acid fraction	253	1,300

* Two aliquots of packed, washed red cells were suspended in equal volumes of *n*-butanol diluted to 0.5 mole per L in either 0.15 M NaCl (Na-loaded RBC) or KCl ("control" RBC). The cells were incubated for 20 minutes at 10° C. Thereafter Na-loaded cells were washed and incubated for 4 hours at 37° C with $^{32}P_i$ in the usual albumin medium of high Na concentration. "Control" cells were incubated similarly in medium of identical composition except for replacement of all but 20 mEq per L Na^+ by K^+. The final intracellular Na^+ content of "control" RBC was 12 mEq per L of RBC; that of Na^+-loaded cells was 17 mEq per L of RBC.

TABLE VI

*Effect of ouabain on the specific activity of phosphatides in HS red cells**

Experiment	Ouabain in medium	Phosphatidic acid fraction	Phosphatidylserine fraction
		cpm/μg P	*cpm/μg P*
1	−	9,970	13,500
	+	14,800	15,400
2	−	34,300	36,200
	+	36,500	39,500

* Two aliquots of the same red cell suspensions were incubated in parallel with $^{32}P_i$ for 4 hours at 37° C in identical suspending media except for the presence of 5×10^{-5} M ouabain. Experiments 1 and 2 represent cells from two HS donors.

Effect of ouabain on phospholipid metabolism in red cells

The cardiac glycoside, ouabain, depresses active pumping of cations in biological membranes by depressing membrane ATPase activity (2). This is the result of the inhibition by ouabain of the dephosphorylation of intermediates that were originally phosphorylated through the agency of ATP (45–47). This dephosphorylation step appears essential to ATPase activity and also to the transport process. One might predict that blockade of the dephosphorylation step by ouabain

might lead to an increased level of radioactivity in the phosphate moiety of intermediates involved in active transport. The specific activity of both phosphatidic acid and phosphatidylserine fractions in HS red cells incubated with $^{32}P_i$ was slightly enhanced by ouabain as shown in Table VI. Similar effects on normal red cells and other tissues have been previously reported (11, 48–50).

Discussion

The molecular basis of active "uphill" transport of cations through biological membranes has been intensively investigated and recently reviewed (1, 2). It has been conclusively demonstrated that this process in red cells is energized by ATP (51–54), through an ATPase system which, in turn, is regulated by the concentrations of intracellular sodium and extracellular potassium ions (15, 55). It is also generally accepted that cations are transported across membranes through the agency of carrier molecules, which bind them on one side of the membrane, ferry them to the opposite side, and there discharge them (1). The possible involvement of phospholipids in this process has been repeatedly suggested because they are constituents of membranes and because they are able to bind cations in the lipid milieu of membranes (1, 2–4, 8–10). The results of the present studies, which demonstrate alterations in phospholipid metabolism concomitant with sodium transport in red cells, are consistent with this suggestion. Indeed, the incorporation of $^{32}P_i$ into membrane phosphatides has been found to be enhanced in a variety of tissues in which transport of cations (11), as well as hormones (6), enzymes, and other proteins (56), or particulate matter (7) has been transiently provoked. In our studies incorporation of $^{32}P_i$ into the phosphatides of HS red cells was roughly twice normal, which coincides with the nearly twofold increment in active transport of sodium ions in these cells (19, 20). The acidic phosphatides, in the phosphatidylserine fraction, and less consistently, the phosphatidic acid fraction, were mainly involved in this hypermetabolism. The present studies also demonstrated that when alterations in the flux of sodium were artificially induced in red cells, labeling of membrane phosphatides was concomitantly altered. Thus, the radioactivity of the phosphatidylserine and phosphatidic acid fractions was decreased toward normal in HS red cells whose sodium fluxes had been reduced by suspending them in media of low sodium content (Table III). Osmotically swollen red cells, whose flux of cations is increased, exhibited an increased labeling in their phosphatidylserine (but not their phosphatidic acid) fraction (Table IV). Furthermore, Na-loaded red cells, which have previously been shown to have increased ATPase (44) and Na-pumping activities (43), incorporated increased amounts of $^{32}P_i$ into their membrane phosphatides (Table V).

Although these studies demonstrate that $^{32}P_i$ incorporation into the acidic phosphatides of red cells can be roughly correlated with flux of cations, they do not allow conclusions regarding the mechanism or mechanisms by which phosphatides may be involved in transport phenomena. It seems unlikely that phosphatidylserine or phosphatidic acid acts directly as a cation carrier in biological membranes. The metabolic sequence of incorporation and loss of phosphate in phosphatidylserine of red cells is not specifically known, and detailed calculations of turnover rates of the phosphate moiety in this compound cannot be made. Nevertheless, the uptake of phosphate by this fraction (roughly 10 μmoles per L of cells per hour) seems orders of magnitude removed from that required if this phosphate group were utilized for direct cation binding and transfer. For instance, it has been estimated that roughly 1,000 μmoles of phosphate per L of cells per hour must be turned over to support the established flux of 3 mEq of sodium ions per L red cells per hour (16). This serious discrepancy in the stoichiometry of cation transport and phospholipid metabolism has been noted also in other tissues and has led to the suggestion (11) that acidic phosphatides, rather than directly carrying cations, alter the conformation, and hence the cation avidity, of other molecules, perhaps proteins (57, 58), which act as the actual cation carriers. This suggestion is strengthened by recent evidence of Tosteson, Cook, and Blount (59) that ATPase activity involved in cation transport can be found in a proteinaceous fraction from sonically disrupted red cell membranes. This fraction contains the bulk of membrane phosphatides. Regardless of what mechanism might be involved, it

would appear from the present studies that acidic phosphatides do have a role in the movement of cations in red cells. A similar conclusion has been reached recently by Ohnishi and Kawamura from evidence that red cell ATPase that has been inhibited by a snake venom phospholipase can be reactivated specifically and solely by replacement of phosphatidylserine (60). On the other hand, it should be noted that in some systems other than red cells, such as the toad bladder (61) and the electric organ of the eel (62), the metabolism of acidic phosphatides does not correlate with cation transport. In this respect, our studies do not allow us to conclude whether changes in phosphatide metabolism are linked to active or to passive transfer of cations, or to both. In each of the circumstances in which increased labeling of phosphatides was noted, both passive flux and active transport of cations were increased.

Our finding that $^{32}P_i$ of the external medium was incorporated into the membrane phosphatidylserine fraction to a significant degree is at variance with some previous reports (63, 64) and in agreement with others (65). Differences in incubation procedures, activities of $^{32}P_i$, and especially the choice of suspending media may explain these discrepancies. We used buffered human albumin solutions as suspending media to circumvent variations in the behavior of different plasma samples (66) and to eliminate such phenomena as plasma-red cell exchange of phosphatides (26, 27). The over-all incorporation of $^{32}P_i$ into red cell phosphatides was suppressed nearly tenfold when we used plasma as the incubation medium rather than buffered human albumin.

It is also possible that other phosphatides, besides phosphatidylserine and phosphatidic acid, might be present in tiny amounts in red cells and be of high radioactivity, yet not be recognized by our chromatographic techniques. We have especially sought for phosphatidylinositides since the turnover of this class in various tissues has been shown to be altered concomitantly with alterations in membrane function (17). Recently Kirschner and Barker (67) have offered evidence in swine red cells that a phosphatide—probably a polyphosphoinositide—incorporates $^{32}P_i$ markedly from suspending media and contaminates the phosphatidic acid separated by column chromatography. Using inositol-labeled phosphatidylinositol as a reference chromatographic standard, we have been unable to document labeling of monophosphatidylinositol from $^{32}P_i$ in red cells under our conditions. Indeed the presence of this compound in human red cells is conjectural (28, 33). Little attention has been paid to zone 1, in which polyphosphoinositides would be found, because this zone did not respond in any striking way to various experimental conditions.

With our chromatographic techniques, the identification of the highly labeled phosphatides as being solely phosphatidylserine and phosphatidic acid should thus not be considered as conclusive. Further, because the amount of phosphatidic acid in red cells is small (28), contamination with other phosphorus-containing lipids, such as polyglycerol phosphatides, might affect the observed specific activity of the phosphatidic acid fraction markedly. The absolute specific activities of phosphatidic acid in these studies thus remain in doubt. On the other hand, the changes in specific activity of the phosphatidic acid fraction with altered cation flux for any given batch of cells whose lipids were extracted and separated under rigidly standardized conditions are, in our belief, secure.

HS red cells manifest increased glucose (19) and ATP (68–70) catabolism, which has been shown to relate to their heightened active transport of sodium (19). It is possible that the increased metabolism of phospholipids we observed in these cells reflects simply this general hypermetabolism. This possibility is thought unlikely for the following reasons: *1*) All membrane phosphatides in HS red cells are not of increased radioactivity. In fact, phosphatidic acid, whose phosphate group is probably derived directly from ATP (71), was not labeled excessively in HS red cells in three of six experiments (Table II) despite increased labeling of the total phosphatide pool of these cells. *2*) The increased metabolism of the acidic phosphatides of HS cells is further enhanced after exposure to ouabain (Table VI) despite the fact that this glycoside renders these cells eumetabolic (19, 70). *3*) Osmotic swelling of red cells, which increases their glucose catabolism (19), consistently increased the labeling of the phosphatidylserine fraction (Table IV), yet often diminished the uptake of $^{32}P_i$ into the total red cell phospholipid pool.

In our preliminary communication (22), we sug-

gested that the increased phospholipid metabolism of HS red cell membranes might be involved both in the abnormal shape and the propensity to undergo osmotic hemolysis. If abnormally rapid turnover of HS phospholipids is associated with decreased avidity of their attachment to the membrane structure, an accelerated loss of surface lipid material from these cells might ensue. The resulting diminution in surface area with constant cellular volume would necessarily result in a more spheroidal cell. Indeed, studies by Prankerd (68) and more recently by Weed, Bowdler, and Reed (72) have demonstrated losses of membrane lipids from HS red cells during incubation *in vitro*. Jacob has confirmed these observations and has presented evidence that this lipid instability is related to the accelerated flux of sodium in HS red cells, for it is completely prevented when flux of sodium is made normal (73). Thus the defect in HS membranes that increases the permeability of the red cell to sodium might not only jeopardize the integrity of the cell by directly increasing the tendency for osmotic swelling and lysis, but also might increase the tendency to spheroidicity by provoking the depletion of membrane phospholipids (73). This depletion is conceivably linked to the increased labeling of phosphatides observed in the experiments reported above, but no mechanistic explanation is yet available.

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