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Research Article

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The effects of stimulation and inhibition of active cation transport, metabolic depletion, and extracellular phosphate concentration on both the degree of labeling and the actual turnover of PA phosphate were studied. In any given experiment, the degree of labeling of PA depended on the specific activities of the other intracellular phosphates (P_i and ATP). The actual turnover rate of PA phosphate, however, did not vary with active transport or metabolic depletion. The greater turnover of PA phosphate in HS erythrocytes may be due to the somewhat younger age of these cells. The results suggest […]

Incorporation of Orthophosphate-^{32}P into Erythrocyte Phospholipids in Normal Subjects and in Patients with Hereditary Spherocytosis

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ABSTRACT The in vitro incorporation of inorganic ^{32}P into erythrocyte phospholipids has been studied in normal subjects and in splenectomized patients with hereditary spherocytosis (HS). Phosphatidic acid (PA) was the only lipid measurably labeled in both kinds of cells. The actual turnover rate of PA phosphate was determined by simultaneously isolating inorganic phosphate (P_i) and adenosine triphosphate (ATP) and determining their specific activities. This turnover is very small: 1.3 μmoles P/liter of erythrocytes per hr in normal cells and 4.0 μmoles P in HS erythrocytes when either ATP or cellular P_i is considered the immediate precursor. This value represents less than 0.1% of the total membrane lipid phosphate. Incorporation of added $^{32}P_i$ into the other phosphatides, including phosphatidyl serine, was essentially zero in both kinds of cells.

The effects of stimulation and inhibition of active cation transport, metabolic depletion, and extracellular phosphate concentration on both the degree of labeling and the actual turnover of PA phosphate were studied. In any given experiment, the degree of labeling of PA depended on the specific activities of the other intracellular phosphates (P_i and ATP). The actual turnover rate of PA phosphate, however, did not vary with active transport or metabolic depletion. The greater turnover of PA phosphate in HS erythrocytes may be due to the somewhat younger age of these cells. The results suggest that the very low turnover of PA phosphate in erythrocytes is mediated by nonspecific enzyme reactions, and that it is quantitatively insignificant in both normal and HS erythrocytes. The results also emphasize the importance of measuring intracellular phosphate precursors in any study evaluating cellular phospholipid turnover from added $^{32}P_i$.

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INTRODUCTION

Although it now seems unlikely that any phospholipid is directly involved as a phosphorylated intermediate in the Na^+-, K^+-adenosine triphosphatase (ATPase) system, many studies continue to show some correlation between phospholipid labeling with ^{32}P and active transport processes in a variety of tissues (1). The majority of studies on the incorporation of orthophosphate-^{32}P into the phospholipids of normal human erythrocytes have indicated that most of the radioactivity is found in phosphatidic acid (PA) (2–6). The Hokins (7, 8) have demonstrated the presence of a diglyceride kinase and PA phosphatase in erythrocyte membranes and have suggested some participation of a PA cycle in active transport in this as well as other cells. Labeling and turnover of phosphate monoesters in erythrocyte inositol phosphatides (PI) has also been reported by this group (9). More recently, Jacob and Karnovsky (10) have suggested that all acidic erythrocyte phospholipids, phosphatidyl serine (PS), as well as PA and PI

become labeled when red blood cells (RBC) are incubated with inorganic ^{32}P and that the turnover of PS phosphate, in particular, is correlated with active cation transport. They report that the labeling of PS is increased by a factor of about two in the erythrocytes of patients with hereditary spherocytosis (HS RBC). They suggest that this increase is due to the greater rate of Na^+ transport in these cells, and that this increased phospholipid turnover is responsible for the loss of lipid and membrane fragmentation found in HS RBC (11–13).

Actual rates of phospholipid turnover have not been determined in the above studies. In the present work, the labeling of all phospholipids by added orthophosphate-^{32}P in normal and HS RBC has been measured serially in vitro. The specific activities of extracellular inorganic phosphate (P_1), intracellular P_i, and ATP were determined simultaneously. This permitted direct calculations of lipid phosphate turnover assuming either P_i or ATP as the immediate precursor. The effect of stimulation and inhibition of Na^+ transport, metabolic depletion, and extracellular phosphate concentration on both the degree of labeling and the actual turnover of erythrocyte lipid phosphate was studied. The results indicate that PA is the only lipid clearly labeled in both kinds of RBC, and that the turnover of this phosphate is very small and of questionable physiologic significance.

METHODS

Blood was collected into Na_2 ethylenediaminetetraacetate (EDTA) (1.25 mg/ml) from normal subjects and from patients with classical HS who had undergone splenectomy. The diagnosis had been established in each case by the appearance of the stained peripheral blood smear and bone marrow, characteristic autohemolysis, and osmotic fragility, the expected familial incidence and virtual correction of the hemolytic anemia after splenectomy.

The blood was centrifuged at 2000 *g* for 15 min at 4°C, with sterile technics and glassware. The plasma was saved and the buffy coat removed as completely as possible. The packed RBC were then resuspended in their plasma, or other media as described below, at a hematocrit of 30–40%. Orthophosphate-^{32}P (10 μc/ml of incubation mixture) and glucose (final concentration 12 mmoles/liter) were added to the plasma before mixing. Within 1 min an aliquot of the incubation was taken and centrifuged at 4°C. This constituted the time zero sample. The incubations were carried out in polyethylene flasks at 37°C in a Dubnoff metabolic shaker with air as the atmosphere. At the end of each incubation period the plasma was removed by centrifugation and the cells were washed three times with about 5 volumes of 0.17 M NaCl at 4°C. The residual white blood cell count was less than 500 cells/mm^3, and platelets were absent from the washed erythrocyte suspensions. In three experiments, the buffy coat was purposely not removed before lipid extraction. This produced no measurable increase in the relative specific radioactivity of PA. A similar finding has been reported by Westerman and Jensen (5). The total lipids were extracted as described previously (14). The individual phosphatides were separated by chromatography on silicic acid–impregnated paper (14) and by column chromatography with silicic acid or diethylaminoethyl (DEAE)-cellulose as described by Rouser, Kritchevsky, and Yamamoto (15). The usual procedure with DEAE-cellulose was to chromatograph about 2.5 mg of total lipid P on a 1×12.5 cm column of 1.5 g. The general elution scheme described by Rouser et al. was followed. This consisted of nine fractions: (*a*) 65 ml of chloroform (C); (*b*) 60 ml of chloroform-methanol (CM) 9:1; (*c*) 60 ml of CM 7:3; (*d*) 60 ml of CM 1:1; (*e*) 65 ml of M; (*f*) 65 ml of C acetic acid 3:1; (*g*) 65 ml of acetic acid; (*h*) 25 ml of M; (*i*) 75 ml of CM 4:1 to which was added 1.5 ml of concentrated ammonium hydroxide and 0.085 ml of 9 M ammonium acetate. The erythrocyte phosphatides were all present in fractions 2, 3, 6, and 9 (Fig. 3). These compounds were identified by their elution position after column chromatography and by their mobilities and staining characteristics with Rhodamine 6-G, ninhydrin, and choline reagents after paper chromatography. Erythrocyte PA was characterized in more detail as described below.

Phosphorus was determined by Bartlett's method (16). Lipid esters were determined by the method of Rapport and Alonzo (17) with methyl stearate as the standard. Glycerol was determined by a modification of the method of Hanahan and Olley (18). The lipid samples were first digested in 0.5 N HCl at 110°C for 72 hr in sealed tubes to insure the complete hydrolysis of glycerophosphate to glycerol and phosphoric acid. Standard solutions of glycerophosphate and glycerol were used in each case to monitor the effects of the digestion. The mean and range of the glycerol to P ratios obtained with glycerophosphate were 0.96 (0.91–1.02), $n = 28$. The mean recovery of glycerol was 100% (97–104%), $n = 22$.

Extracellular P_1 was isolated by ultrafiltration of the plasma (19). Trichloroacetic acid (TCA) extracts were prepared by diluting washed RBC suspensions in 19 volumes of 8% TCA. Intracellular P_1 and ATP were isolated from these neutralized extracts by anion-exchange resin and charcoal chromatography as described by Bartlett (20). Cellular Na^+ content was measured by flame photometry after washing the RBC with a (4°C) solution containing 0.1 M $MgCl_2$ and 0.05 M Tris buffered at pH 7.4 (12).

The specific activities (SA) of the column fractions and individual lipid spots after paper chromatography were determined with a low-background, thin-window, gas-flow detector system as described previously (21, 22). The labeling of the compounds studied is reported as

their relative specific activities (RSA). This was obtained, as a per cent, by dividing the SA of a given compound by the SA of extracellular P_i at time zero and multiplying the quotient by 100 (21). A compound was considered unlabeled if its RSA was less than 0.005%. At least 500 counts were accumulated to reach this decision.

RESULTS

Fig. 1 is a radioautograph of normal erythrocyte lipids after incubation with orthophosphate-^{32}P for 6 hr. The only visibly labeled compound is PA which constitutes only 3% of the total lipid P. Fig. 2 shows the results of fractionation of larger amounts of normal RBC lipids by silicic acid column chromatography after 6 hr of incubation with the isotope. About 90% of the radioactivity was recovered in the fraction eluted with 5% methanol in chloroform. This fraction consisted of a single lipid having the mobility of PA on paper chromatography. The glycerol to P to ester ratio of the material in this fraction was 1.0:1.0:1.95 (theory for PA, 1.0:1.0:2.0). These findings indicate that the labeled erythrocyte lipid is in fact PA and not a polyglycerol phosphatide.

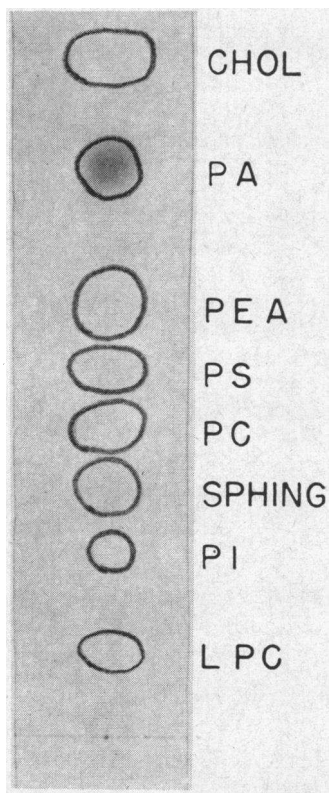

FIGURE 1 Radioautograph of total lipids from normal erythrocytes after incubation with ^{32}P for 6 hr. About 3.0 μg of total lipid phosphorus was chromatographed on silicic acid–impregnated paper with a solvent system of diisobutylketone, *n*-butyl ether, acetic acid, and water 20:20:20:3 (v/v), at 4–6°C. The lipid spots were outlined under ultraviolet light after staining with Rhodamine 6-G before radioautography. *Chol,* cholesterol; *PA,* phosphatidic acid; *PEA,* phosphatidyl ethanolamine; *PS,* phosphatidyl serine; *PC,* lecithin; *Sphing,* sphingomyelin; *PI,* phosphatidyl inositol; *LPC,* lysolecithin. *LPC* is a minor and inconstant component of erythrocyte lipids. The size of the spot does not correspond to the amount present.

Traces of radioactivity were also found in the fractions eluted with 10% and 50% methanol in chloroform as well as with methanol alone. In each instance paper chromatography showed small amounts of labeled PA in these fractions and this compound accounted for all the radioactivity present. Paysant-Diament and Polonovski (2) also found that small amounts of erythrocyte PA were eluted from silicic acid columns with solvent mixtures more polar than 5% methanol in chloroform, which elutes most of the material. This may be due to the presence of salts of PA which bind more tightly to the column than the acid form.

When HS RBC were incubated with $^{32}P_i$ the same qualitative results were obtained: PA was the only lipid to become clearly labeled. Fig. 3 illustrates fractionation of HS erythrocyte lipids with the other column technic used, chromatography on DEAE-cellulose. With this method all species of PA are eluted as the acid form with a solvent containing acetic acid.

With this combination of column and paper chromatography, the RSA of all the phosphatides in normal and HS RBC were determined serially over a 6 hr period in vitro. In all experiments with both kinds of cells, at the end of 6 hr, the RSA of PI was less than 0.01%, and the RSA of lecithin (PC), sphingomyelin, PS, and PEA were less than 0.005%.

Fig. 4 shows the results of experiments in which the RSA of PA from normal erythrocytes was determined at frequent intervals beginning at 5 min. The labeling increases linearly over the 8 hr period. This indicates that erythrocyte PA phosphate behaves as a uniform pool over this interval.

The fractional turnover per hour (k) of PA phosphate in normal and HS RBC was calculated from the data given in Table I (23). The value for k is approximately 0.01 in normal cells and

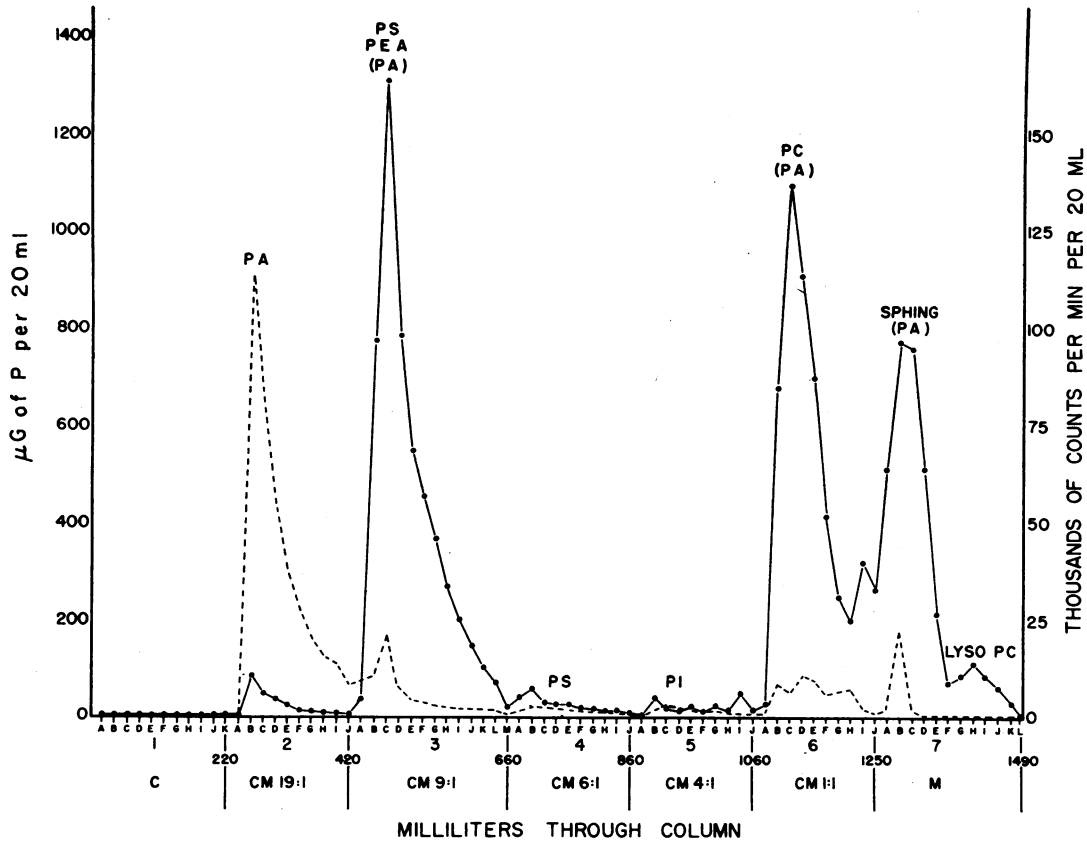

FIGURE 2 Silicic acid column chromatography of normal erythrocyte lipids after incubation with ^{32}P for 6 hr. About 15 mg of total lipid phosphorus was chromatographed on a 1.5 × 15 cm column of silicic acid (20 g) at 4–6°C with the indicated solvents. *C*, chloroform, *M*, methanol. Solid curve, phosphorus; broken curve, radioactivity in counts per minute. Abbreviations for the lipids are as in Fig. 1. The phospholipid content of each tube (20 ml) was determined by paper chromatography.

0.03 in HS RBC. Essentially the same values are obtained whether ATP or P_i is considered the immediate precursor. The low fractional turnover of the small PA phosphate pool amounts to about 1.3 μmoles P/liter of RBC per hr in the normal cell and 4.0 μmoles of P in HS RBC. This represents a turnover of less than 0.1% of the total membrane lipid P (4.5 mmoles/liter of RBC) even in the HS RBC.

Because of the suggestions that the turnover of phosphate in PA or other membrane phosphatides may be correlated, at least, with active cation transport this question was examined in the present study. Cellular Na^+ loading and stimulation of transport was achieved by using the "cold-stored" cell (24). Blood was stored in acid citrate dextrose (ACD) solution (NIH formula A) at 4°C for 7 days. The blood was then centrifuged at 2000 *g* at 4°C for 15 min and the ACD plasma removed. The cells were washed once and then resuspended in fresh plasma with glucose and orthophosphate-^{32}P and incubated for 6 hr at 37°C. In these experiments the mean Na^+ content of the cells was 8.9 mEq/liter of RBC before storage, 17.5 mEq/liter of RBC after the 7 day period of cold storage, and 10.7 mEq/liter of RBC after the 6 hr period at 37°C. Thus the average rate of active Na^+ transport was increased by about 40% over the 6 hr period; it was about doubled during the first 3 hr. The results of these experiments are shown in Table II. At 6 hr, the RSA of the other phosphatides were essentially zero, as before. The RSA of P_i was somewhat increased and higher than that of ATP in contrast to the findings in the fresh cell. This may reflect some initial increased permeability to phosphate ions in the

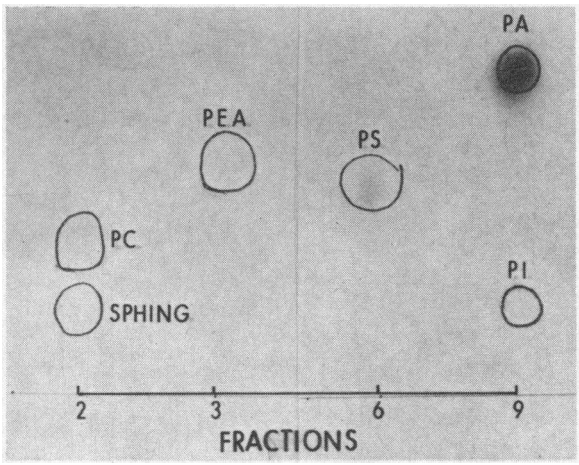

FIGURE 3 Radioautograph of lipids from hereditary spherocytosis erythrocytes (HS RBC) separated by diethylaminoethyl (DEAE)-cellulose column chromatography. 2.5 mg of total lipid P from HS RBC incubated with ^{32}P for 6 hr was chromatographed on a 1 × 12.5 cm column of DEAE-cellulose (1.5 g). The nine fractions described in the Methods section were obtained and chromatographed on silicic acid-impregnated paper. The phosphatides were all present in the four numbered fractions shown. *PA* is clearly labeled. A trace of radioactivity may be present with the *PS* spot. The amount of *PS* was purposely made 10 times greater than *PA* on this chromatogram. The RSA of *PS* was 0.004%; that of *PA* was 1.5%. Abbreviations are as in Fig. 1.

stored RBC. The RSA of PA was normal and this indicates that the stimulation of Na^+ transport did not cause a significant increase in PA phosphate turnover.

The effects of inhibition of active cation transport were studied by (1) the addition of 1×10^{-4} M ouabain to the standard incubation, and (2) by incubating normal erythrocytes in a low Na^+ medium consisting of 1 mM phosphate buffer, 10 mEq of Na^+ per liter, 130 mEq of choline per liter, and Cl^- for the balance of the anions. The results of these experiments are shown in the first two columns of Table III. With ouabain, the RSA of PA was somewhat lower but still within the normal range. The RSA of PA is normal or slightly increased in the low Na^+ medium. These results indicate that inhibition of active cation transport does not materially affect the turnover of PA phosphate.

Grossman, Horky, and Kohn (25) have reported that plasma may inhibit the incorporation of orthophosphate-^{32}P into erythrocyte phospholipids, and that a saline medium has a stimulating effect. In these studies the degree of labeling was measured as the per cent of added radioactivity incorporated. Column 3 of Table III shows the results obtained in the present study when erythrocytes were incubated in normal saline with ^{32}P and sufficient carrier phosphate (about 1 μmole/ml) so that the actual SA of extracellular phosphate could be determined easily. The RSA of PA are the same as those found in plasma, and this indicates that the presence of plasma proteins does not inhibit the labeling of erythrocyte phosphatides when this is calculated on the basis of the SA of extracellular phosphate at time zero.

The effect of metabolic depletion on the incorporation of $^{32}P_i$ into erythrocyte phospholipids was also studied. Normal RBC were washed three times with 0.17 M NaCl, which removed cellular glucose, and then incubated for 12 hr at 37°C in plasma that had been made free of glucose by dialysis against a 1 mM phosphate buffer. The cells were then recovered by centrifugation, resuspended in glucose-free plasma containing $^{32}P_i$, and incu-

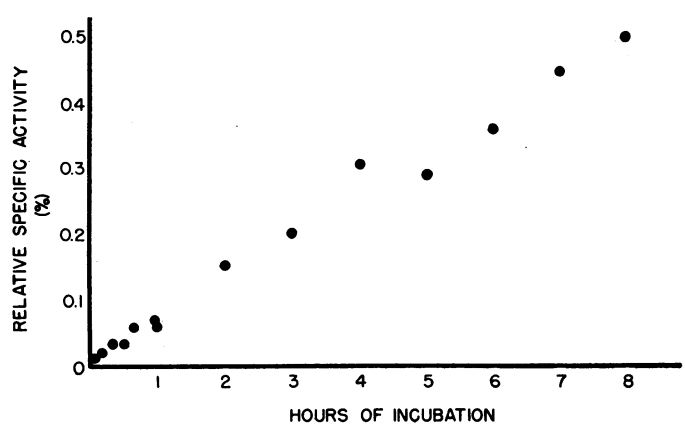

FIGURE 4 Serial determinations of the relative specific activity (RSA) of phosphatidic acid (PA) from normal erythrocytes (RBC). Normal RBC were incubated in plasma with ^{32}P at 37°C. The RSA of PA was determined serially from 5 min to 8 hr. PA was isolated by chromatography on silicic acid-impregnated paper. The findings shown are the results of four experiments each covering separate portions of the 8 hr interval. It can be seen that the RSA of PA increases linearly over this period.

TABLE I

RSA of Erythrocyte Phosphate Compounds during In Vitro Incubations with ^{32}P in Five Normal Subjects and Four Patients with HS

Incubation time	ATP		P_i		PA	
	Normal	HS	Normal	HS	Normal	HS
hr						
1/2	4.2 (3.1–5.1)*	3.5 (2.9–4.4)	3.0 (2.3–5.1)	2.8 (2.2–4.2)	0.04 (0.01–0.05)	0.09 (0.06–0.11)
1	5.6 (4.5–6.6)	5.2 (4.1–6.5)	3.4 (2.5–3.9)	3.2 (1.8–5.6)	0.09 (0.07–0.11)	0.20 (0.18–0.22)
2	7.5 (6.0–9.3)	6.2 (4.6–7.9)	5.6 (4.4–7.7)	4.1 (2.1–5.7)	0.18 (0.10–0.30)	0.51 (0.49–0.52)
4	9.3 (8.1–11.1)	8.7 (6.6–10.7)	7.5 (6.9–8.0)	5.8 (3.6–7.1)	0.44 (0.25–0.70)	1.06 (0.95–1.15)
6	9.5 (7.6–11.3)	9.5 (7.0–12.2)	7.9 (6.8–9.8)	6.8 (4.7–9.1)	0.56 (0.39–0.90)	1.52 (1.13–1.85)

RSA, relative specific activity; HS, hereditary spherocytosis; ATP, adenosine triphosphate; P_i, inorganic phosphorus; PA, phosphatidic acid.

* Mean values, ranges in parentheses.

bated at 37°C for an additional 6 hr. The results are shown in Table IV. The RSA of intracellular P_i were very high and exceeded those of ATP. This, again, is probably due to a marked increase in permeability to phosphate ions in these metabolically depleted erythrocytes. Very small amounts of ATP were found (less than 0.3 μmole/ml of RBC) but the RSA of this material was two to three times greater than normal. The RSA of PA was also increased by a factor of 2 to 3. This apparent stimulation of PA labeling probably merely reflects the higher RSA of the immediate intracellular precursor phosphate. Table IV also shows the results obtained when normal erythrocytes were incubated in plasma in which the final phosphate concentration had been raised to 10 mmoles/liter. This produced a substantial increase in the RSA of PA, but there was also a corresponding increase in the RSA of ATP and P_i. These results suggest that any circumstance producing a substantial increase in phosphate flux may cause increased labeling of cellular lipids with $^{32}P_i$, but that this does not represent greater phospholipid turnover.

TABLE II

Effect of Increased Active Na^+ Transport on RSA of Phosphate Compounds in Cold-Stored RBC during Poststorage Incubation at 37°C

Incubation time	ATP	P_i	PA
hr			
2	6.3*	9.2	0.18
4	7.4	9.4	0.36
6	8.5	10.4	0.51

Abbreviations same as in Table I.

* Mean values of two experiments.

DISCUSSION

In the present study, when a combination of column and paper chromatography for lipid isolation was used, PA was the only phosphatide found to become quantitatively labeled from added $^{32}P_i$ in both normal and HS RBC. Although the labeling of PI was extremely small, it may have been somewhat greater than that of the other phos-

TABLE III

Effect of 10^{-4} M Ouabain, Low Na^+ Medium, and Normal Saline on the RSA of PA in Normal Erythrocytes

Incubation time	Ouabain	Low Na^+	Normal saline
hr			
2	0.12	0.32	0.21
4	0.25	0.65	0.53
6	0.40	0.98	0.70

Abbreviations same as in Table I.

TABLE IV

Effect of Prestorage at 37°C without Glucose and of 10^{-2} M Phosphate on the RSA of Normal Erythrocyte Compounds

Incubation time	Prestorage			10^{-2} M phosphate		
	ATP	Pi	PA	ATP	Pi	PA
hr						
2	17	27	1.1	29	46	0.55
4	16	29	1.8	31	50	1.2
6	24	37	2.2	33	50	1.8

Abbreviations same as in Table I.

phatides (RSA of PI less than 0.01%; RSA of the others less than 0.005%). Hokin and Hokin (9) have shown that erythrocyte ghosts can incorporate ^{32}P from labeled ATP into the monoesterified phosphates of polyphosphoinositides. The amount of di- and triphosphoinositides in erythrocytes is probably quite small. Wieneke and Woodin (26) have estimated that these lipids constitute about 0.5% of the total lipid P, or only 1/6th of the inositol phosphatides present in erythrocytes. Some labeling of these trace components may have accounted for the slightly higher RSA of PI in the present experiments. The low turnover of lipid phosphate monoesters as are present in PA and the polyphosphoinositides can be simply mediated by the nonspecific action of a kinase or the reversal of a phosphatase reaction. Such enzymes have been found in erythrocytes (7, 8). On the other hand, the introduction of ^{32}P into lipid phosphodiesters, found in the other phosphatides, would require a more complicated biosynthesis of the lipid molecule. The necessary enzymes for this type of process have not been found and true lipid synthesis from simple procursors does not occur in the mature red blood cell (27).

The actual PA turnover rate of only 1.3 μmoles P/liter of RBC per hr found in the present study indicates that this phosphate cannot serve as an intermediate in the Na^+-, K^+-ATPase reaction in the red blood cell. A similar conclusion has been drawn in other tissues from the wide discrepancies between the SA of ATP and phospholipids observed in labeling experiments (28–30). The exact significance or role that this turnover may have is not clear at this time. The present results indicate the necessity of measuring the SA of ATP and other intracellular phosphates when evaluating concomitant increased lipid labeling and stimulation of active transport or other processes. In the metabolically depleted cell where virtually no active transport occurred there was a substantial stimulation in the labeling of PA. As indicated above this reflected the higher RSA of the intracellular phosphates and the actual turnover was probably unchanged. The same findings obtained in the high plasma phosphate experiments. Na^+ loading in the "cold-stored" cell was used in the present study to measure the effect of increased transport on the labeling of PA because, as shown in Table II, the RSA of ATP were the same and the RSA of P_i only slightly greater than those in unstored cells. No concomitant rise in PA phosphate turnover was found under these circumstances. Some technics used to stimulate active transport may at the same time alter membrane permeability to phosphate or otherwise increase the labeling of intracellular ATP and P_i. This may account for the correlation between transport and lipid labeling that has been found in the past.

The greater labeling of PA in HS RBC is not due to higher RSA of intracellular phosphates. The actual turnover was increased 3-fold to a level of about 4.0 μmoles P/liter of RBC per hr. This still represents less than 0.1% of the membrane lipid P. Jacob and Karnovsky (10) found that labeling of PS rather than PA was increased two to three times in HS RBC, and that labeling of PS, in particular, was correlated with the rate of active Na^+ transport. We have no explanation for the difference between these results and our own, in which the RSA of PS was consistently less than that of PA by a factor of at least 100 in all experiments with both normal and HS RBC. Our failure to find any correlation between active transport and the actual turnover of PA phosphate suggests that increased Na^+ pumping is not the cause for this greater turnover in HS RBC. Another possibility is that this is due to the somewhat younger red cell population in our patients with HS, whose reticulocyte counts ranged from 2 to 4%. To date we have found a similar increase in the RSA of PA in one patient with reticulocytosis secondary to acquired hemolytic anemia. Further work to explore this possibility is in progress.

Jacob (13) has also suggested that the greater phospholipid turnover in HS RBC, as measured by ^{32}P incorporation, is causally related to the loss

of lipid and membrane fragmentation which these cells undergo. Previous work from this laboratory (11, 12) has suggested that premature alteration in the physical properties of the membrane protein in HS RBC resulting from ATP depletion was the critical factor in producing loss of membrane substance in these cells. In the present experiments, glucose deprivation and marked reduction of cellular ATP had no effect on PA phosphate turnover. Moreover, the net increase of this turnover in HS RBC would amount only to about 1% of the membrane lipid P over a period of 24 hr. It is not clear how this might be related to a loss of 20% of the membrane lipid during a 24 hr period, but the present results do not completely exclude this possibility.

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