PHOSPHOLIPID METABOLISM IN INTACT AND MODIFIED ERYTHROCYTE MEMBRANES

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ABSTRACT

Erythrocyte membranes incorporated labeled phosphate from γ-adenosine triphosphate ($AT^{32}P$) into phosphatidic acid and the polyphosphoinositides. Inositol-^{3}H and palmitate-^{14}C were also incorporated into the phospholipids but α-glycerophosphate-^{32}P was not. The incorporation of γ-$AT^{32}P$ into phospholipids was increased when the erythrocyte ghosts were incubated in hypotonic media which lysed the cells. Lysis had little or no effect on the incorporation of inositol-^{3}H and palmitate-^{14}C into the phospholipids.

If erythrocyte membranes were prepared in 1 mM ethylenediaminetetraacetate (EDTA), instead of 1 mM $MgCl_2$, then the tonicity of the incubating medium did not influence the incorporation of γ-$AT^{32}P$ into the phospholipids. Erythrocyte ghosts, prepared by lysis in water, EDTA, or 1 mM calcium, lead, mercury, zinc, or cadmium, failed to reconstitute when placed in isotonic medium, inasmuch as they did not retain potassium against a chemical gradient. Ghosts prepared by lysis in 1 mM magnesium, barium, or strontium could be reconstituted. Ghosts which failed to reconstitute incorporated more labeled phosphate from γ-$AT^{32}P$ into the phospholipids than did intact or reconstituted ghosts. The larger incorporation of labeled phosphate by leaky ghosts was not due to a greater entrance of γ-$AT^{32}P$ into those cells.

Primaquine phosphate and digitonin, at concentrations which are known to cause cells to form smaller vesicles or to lyse cells by removing cholesterol, did not increase the incorporation of labeled phosphate into the phospholipids.

It is suggested that the increased metabolism of phospholipids may be involved in a membrane repair mechanism.

INTRODUCTION

The characteristic ability of the red cell membrane to lyse under a variety of conditions and to reconstitute itself insofar as permeability properties are concerned (1, 2) afford a model for the study of the chemical changes which may occur in a membrane during the processes of membrane damage and repair.

The phospholipids of the human erythrocytes undergo a very slow rate of turnover. It has been demonstrated, however, that human erythrocytes can incorporate labeled inorganic phosphate into phosphatidic acid (3, 4) and also into the polyphosphoinositides (5), although the large majority of phospholipids do not incorporate phosphate. There are some indications that there may be some interchange of complete phospholipid molecules between red cells and plasma, but it is believed that this occurs only to a very limited extent (6). The red cell is capable, however, of incorporating unesterified fatty acids from the external medium into phospholipids. It has been calculated that the extent of phospholipid fatty acid turnover is approximately 2% per hr, and that this represents an expenditure

of about 2% of the energy available from glycolysis (7). Hence, phospholipids, although not so active metabolically in the erythrocyte membrane as in membranes of other animal cells, are still in a dynamic metabolic state. This report describes experiments designed to determine whether changes in phospholipid metabolism accompany the processes of membrane damage and repair.

MATERIALS AND METHODS

Preparation of Red Cells and Membrane Fractions

RED CELLS: Fresh, heparinized human blood was used. Red cells were prepared by centrifuging the blood in the cold at 1570 *g* for 10 min and by washing the cells three times with cold 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.4. Care was taken to remove all of the buffy layer above the red cells

MG^{++}-LYSED MEMBRANES AND RECONSTITUTED "GHOSTS": The washed red cells were mixed with 10 volumes of cold 1 mM $MgCl_2$ or 1 mM $MgCl_2$ in 10 mM Tris, pH 7.4, and centrifuged at 27,000 *g* for 10 min. The pellet was washed three times with 1 mM $MgCl_2$ in 10 mM Tris-HCl, pH 7.4. The resulting washed precipitate was resuspended in 10 mM of Tris-HCl, pH 7.4. This fraction is called the "red cell membranes." In some experiments 15.5 ideal milliosmolar (imOsM) Na phosphate, pH 7.4, was used instead of the 1 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.4, buffer.

Reconstituted ghosts were prepared by the method of Whittam and Ager (8). After the addition of 5–10 volumes of 1 mM $MgCl_2$ to the washed red cells, sufficient 3 M NaCl was added to raise the final concentration of the hemolysate to 0.17 M NaCl. The hemolysate was then incubated at 37°C for 30 min to reconstitute the membranes. The reconstituted ghosts were sedimented at 27,000 *g* for 10 min, washed twice, and resuspended with 0.15 M NaCl in 10 mM Tris, pH 7.4.

EDTA-LYSED MEMBRANES: Washed red cells were lysed by mixing with 10 volumes of 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, for 10 min in the cold. The lysed cells were washed three times with the same EDTA buffer. The centrifuged membranes were white, showing no traces of attached hemoglobin. The EDTA-lysed membrane preparations were then resuspended in the appropriate buffer.

RECONSTITUTED GHOSTS PREPARED AFTER LYSIS WITH DIFFERENT CATIONS: Washed red cells were lysed in 10 volumes of a 1 mM solution of the cation to be tested. The lysed cells were resealed by adding sufficient amounts of a 3 M salt solution (3:1, K/Na) to raise the molarity to 0.17. The resealed ghosts were washed three times with 0.17 M NaCl in 0.01 M Tris-HCl, pH 7.4. The packed, washed, reconstituted ghosts were used for determination of potassium in a flame photometer or for incubation with radioactive precursor.

Materials

Palmitic acid-^{14}C, uniformly labeled (640 mCi/mmole), was purchased from New England Nuclear Corp., Boston, Mass. This radioactive fatty acid was absorbed on human albumin by the method of Shobet et al. (7). The final material contained 5×10^6 cpm of palmitic acid and 23.5 mg albumin in 1 ml of solution.

γ-$AT^{32}P$ was prepared by the method of Glynn and Chappell (9), and α-glycero-^{32}P by the method of McMurray et al. (10). Inositol-2-^{3}H was purchased from New England Nuclear Corp. and had a specific activity of 3 Ci/mmole. Bacterial alkaline phosphatase was obtained from Worthington Biochemical Corp., Freehold, N. J., primaquine phosphate was a gift from the Sterling-Winthrop Research Institute in Rensselaer, New York, and digitonin was of certified grade from Fisher Scientific Company, Pittsburgh, Pa.

Lipid Extraction and Chromatography

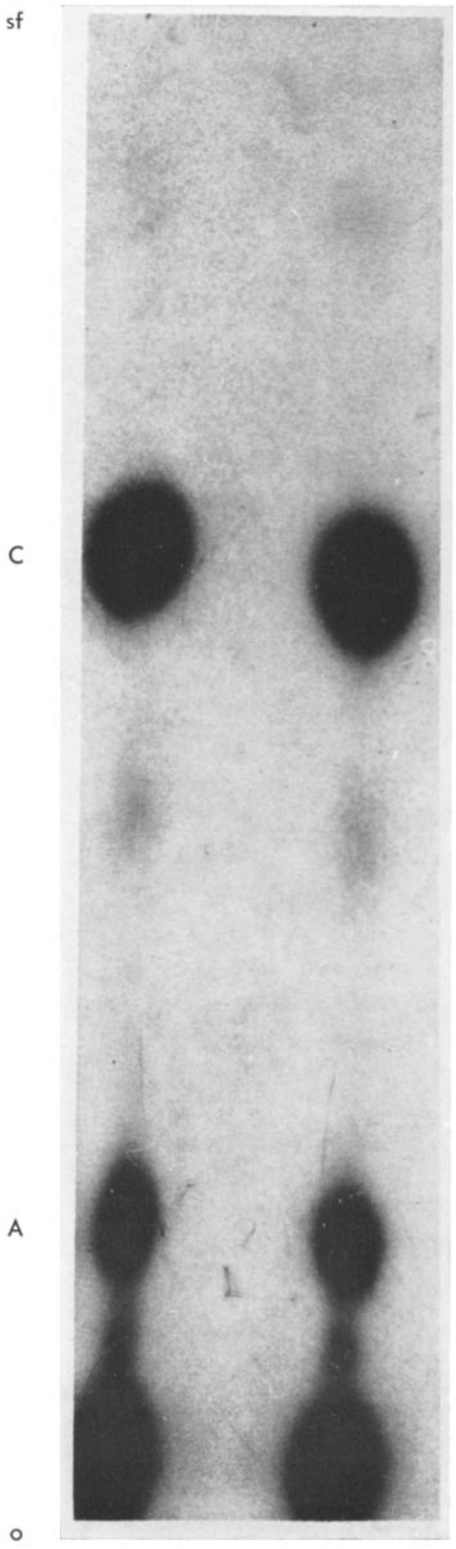
Phospholipids were extracted from the trichloroacetic acid (TCA)-precipitated material by using $CHCl_3$ methanol-concentrated HCl (200:100:1, v/v) as described by Folch (11). Paper chromatography was performed on silica gel-loaded paper with either phenol-NH_3 (15) or diisobutyt ketone–acetic acid–water as solvent (25).

Buffer mixtures of sodium phosphate to give the desired ideal milliosmolarity (imOsM) were prepared as described by Dodge et al. (12). Phosphorus was determined by the method of Bartlett (13). The extracted phospholipids were deacylated as described by Dawson (14), and the deacylated materials were chromatographed on Dowex 1-formate (Dow Chemical Co., Midland, Mich.) as previously described (15) or on paper (16).

RESULTS

Identification of Radioactive Phospholipids

Four radioactive spots were obtained when the chloroform extract, from ghosts incubated with γ-$AT^{32}P$, was chromatographed on silicic acid–impregnated paper, as described by Santiago-Calvo et al. (15). This agrees with the earlier work of Hokin and Hokin who identified these lipids as triphosphoinositide, diphosphoinositide, an unknown material, and phosphatidic acid



(5). Most of the radioactivity obtained under the conditions used in these experiments occurred in two areas with R_f values of 0.19 (band A) and 0.65 (band C). Minor radioactive areas were occasionally noticed at R_f values of 0.09 and 0.50. Sometimes the radioactive area at R_f 0.65 appeared to separate into two spots. Bands A and C were always obtained, and it is these compounds which were routinely counted and identified. Fig. 1 shows a typical chromatogram.

The material at R_f 0.65 (band C) was eluted from the paper and was rechromatographed on silica gel–loaded paper using the diisobutyl ketone:acetic acid:water (40:25:5) solvent system as described by Marinetti (25). It had an R_f value of 0.75, identical with that of phosphatidic acid. No other radioactive spots were observed. The material with R_f 0.19 (band A) streaked in the Marinetti system. This is a property that the polyphosphoinositides exhibit in this system.

The two major radioactive bands (A and C) were deacylated with methanolic sodium hydroxide as described by Dawson (14). The deacylated material was chromatographed in two different paper chromatographic systems and also on Dowex 1–formate. The deacylated material from band C chromatographed with α-glycerophosphate, indicating that the parent lipid was phosphatidic acid. The deacylated material from bands A and B, on chromatography in the system described by Desjobert and Patek (16), gave an R_f value relative to that of α-glycerylphosphorylinositol diphosphate. The minor band with an original R_f of 0.50 gave a deacylated product with an R_f value similar to that of glycerylphosphorylinositol phosphate (5). This indicates that the parent phospholipids are triphosphoinositide and diphosphoinositide.

Ghosts prepared by lysis with cadmium incorporated ^{32}P of γ-AT^{32}P mostly into bands A and B, with little or no incorporation into band C.

FIGURE 1 Autoradiogram showing the chromatographic separation of the ^{32}P-labeled erythrocyte phospholipids. Phospholipids from erythrocyte ghosts incubated with γ-AT^{32}P in Krebs-phosphate buffer without saline were chromatographed on silica gel–impregnated paper using phenol-NH_3 as solvent as described in Materials and Methods. The identification of the radioactive bands *A* and *C* is given in Results. *o* is the origin and *sf* is the solvent front.

When the deacylated chloroform extract from such material was chromatographed on Dowex 1–formate as described by Santiago-Calvo et al. (15), two peaks were obtained. One peak was eluted with 0.4 M ammonium formate in 4 N formic acid, and the other with 0.8 M ammonium formate in 4 N formic acid. Previous work had identified material with this chromatographic behavior on Dowex 1–formate as glycerylphosphorylinositol phosphate and glycerylphosphorylinositol diphosphate (15).

These results indicate that the radioactive phospholipids are phosphatidic acid (band C) and the polyphosphoinositides (band A aad B), and agree with the previous studies of Hokin and Hokin (5) who found these to be the major radioactive products produced when erythrocyte ghosts were incubated with γ-AT^{32}P.

Treatment of the Deacylated Material from Band A with Alkaline Phosphatase

The deacylated material from band A (glycerylphosphorylinositol diphosphate), on treatment with alkaline phosphatase, liberated more than 95% of its radioactivity as inorganic phosphate. Hence, the diesterified phosphate of triphosphoinositide is not labeled by γ-AT^{32}P. This indicates that the majority of the radioactivity in triphosphoinositide is obtained by the phosphorylation of phosphatidylinositol or diphosphoinositide. A similar finding was obtained by Hokin and Hokin (5).

The Incorporation of ^{32}P of γ AT^{32}P into the Phospholipids of Reconstituted Erythrocyte Ghosts

Reconstituted ghosts were used to study the metabolism of membrane phospholipids in reconstituted membranes and in lysed membranes, the latter referring to reconstituted ghosts incubated under hypotonic conditions. Phospholipid metabolism was first measured by following the incorporation of ^{32}P of γ-AT^{32}P into the isolated total phospholipids. In the reconstituted membranes there was only a small amount of incorporation, whereas in the lysed membranes there was a large incorporation of phosphate from γ-AT^{32}P into the phospholipids (Fig. 2). Separation of the phospholipids by chromatography revealed that about 70–75% of the radioactivity was in phosphatidic acid and that the remainder was in the polyphosphoinositides.

Incorporation of phosphate into the phospholipids only occurs at salt concentrations below 0.05 M. At higher salt concentrations the incorporation is similar to that seen in isotonic medium (Fig. 3 A). The amount of hemolysis as measured by the release of hemoglobin from the reconstituted ghosts incubated under these conditions is shown in Fig. 3 B. The reconstituted ghosts lose their entrapped hemoglobin at a salt concentration between 0.05 and 0.09 M.

The Effect of Varying Salt Concentration on the Incorporation of ^{32}P from γ AT^{32}P into Phospholipids of Lysed Membranes

The previous experiments were performed with reconstituted ghosts. These are cells which have been resealed and have regained their semipermeable properties. The increased incorporation of phosphate into the phospholipids may be due to the opening or lysing of the cells. The experiments described in this section were done with cells which were "open" to begin with and

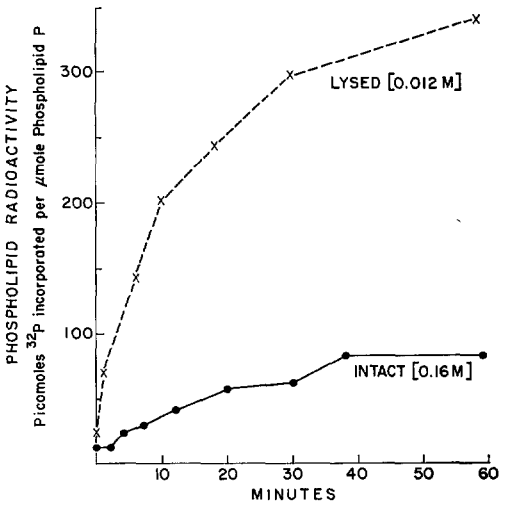


FIGURE 2 Time course of incorporation of γ-AT^{32}P into osmotically lysed and intact erythrocyte membranes. Reconstituted ghosts were prepared as described in the text and were incubated with 0.01 M Tris-HCl, pH 7.4, 1 mM $MgCl_2$, and 0.1 μmole/ml of γ-AT^{32}P (5.2×10^7 cpm/μmole). One set of ghosts were incubated with the above medium plus 0.16 M NaCl, and the other in hypotonic medium with 0.012 M NaCl. Incubation was carried out at 37°C.

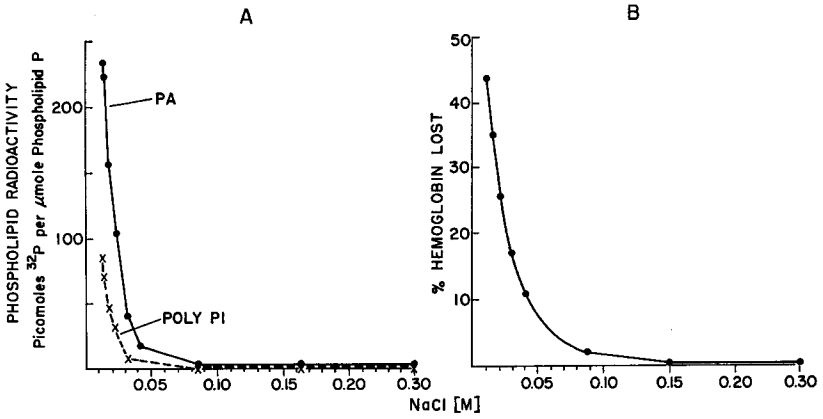


FIGURE 3 Incorporation of γ-AT^{32}P into phospho ipids, and the release of hemoglobin by erythrocyte ghosts incubated with varying amounts of salt. Reconstituted ghosts were incubated at 37°C for 10 min with varying NaCl concentrations under conditions described in Fig. 2. The hemoglobin released was measured by reading the optical density of the released material at 500 mμ. The radioactivity in phosphatidic acid (band C) and triphosphoinositide (band A) was determined as described in the text.

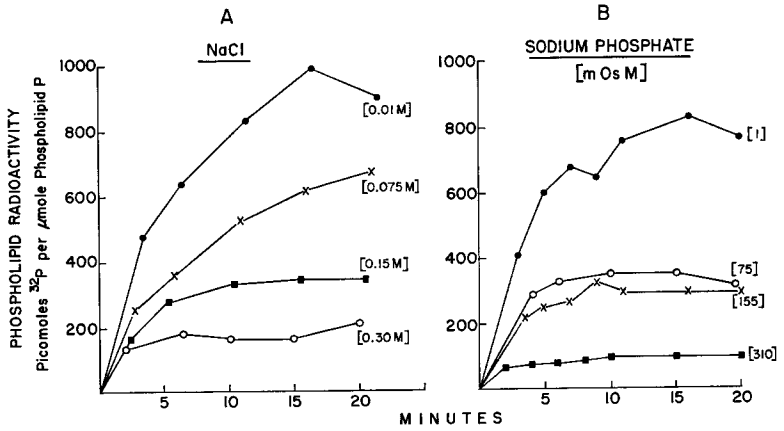


FIGURE 4 Time course of incorporation of γ-AT^{32}P into phospholipids of erythrocyte membranes incubated in solutions of different osmolarities. Magnesium-lysed membranes were prepared as described in Materials and Methods. The membranes were not reconstituted prior to incubation. Experiment A was performed with 0.1 M Tris-HCl, pH 7.4, with 1 mM $MgCl_2$ and 0.1 μmoles γ-AT^{32}P/ml (6×10^7 cpm/μmole), and experiment B with the same concentrations of Mg^{++} and γ-AT^{32}P but with varying concentration of sodium phosphate buffer at pH 7.4.

then were closed or allowed to remain open during incubation with γ-AT^{32}P. This was done to determine whether the phospholipid effect was due to either the osmotic opening or the closing of the membrane.

In a medium in which the membranes do *not* reseal (0.01 M NaCl or 1 mOsM sodium phosphate buffer), there was maximum incorporation into the total phospholipids for about 10–15 min. As the salt concentration was raised and the membranes started to reseal, the amount of incorporation of γ-AT^{32}P into phospholipids decreased (Fig. 4). The type of salt used to change the tonicity of the medium was not critical. Fig. 4 shows two experiments, one using NaCl and the other using phosphate buffer. Both show similar responses to increasing salt concentrations. However, when sodium phosphate buffer was used instead of NaCl there was less incorporation into the polyphosphoinositides, with 90% of the incorporation found in phosphatidic acid.

The Effect of Salt Concentration on Phospholipid Metabolism of "Leaky" Membranes

Erythrocyte ghosts prepared by lysing in the presence of EDTA do not regain their semipermeable properties with respect to Na^+ and K^+ when put into an isotonic medium. Membranes prepared in this manner always remain leaky. The subsequent addition of Mg^{++} and Ca^{++} to cells which were lysed with EDTA fails to repair this damage (9). When "leaky" membranes, prepared by lysis in 1 mM EDTA, were incubated with γ-AT^{32}P, it was found that Mg^{++} was necessary for phosphate incorporation into the phospholipids. In the presence of Mg^{++} there was active incorporation and most of the radioactivity was found in the polyphosphoinositides. Unlike cells prepared by lysis in 1 mM Mg^{++}, the EDTA-lysed cells were not affected by the salt concentration, and they incorporated γ-AT^{32}P into phospholipids equally well in hypotonic or isotonic medium (Fig. 5).

If Ca^{++} was introduced into the medium, the radioactivity was found in phosphatidic acid and not in the polyphosphoinositides, but again there was little or no effect of salt (Fig. 5). Hence, increased phospholipid metabolism is not due to lowering the salt concentration, but is manifested in all leaky cells regardless of the salt concentration.

The Effect of Cations on the Reconstitution of Erythrocyte Membranes and on Phospholipid Metabolism

The ability to reconstitute an erythrocyte membrane is influenced not only by EDTA. The ionic content of the lysing medium is of importance. For example, if cells are lysed at very low calcium concentrations they are able to retain potassium against a chemical gradient but they lose this ability if they are lysed at higher calcium concentration. This is in contrast to cells lysed in magnesium which readily regain their semipermeable properties when placed in isotonic medium (17). Thus, it is possible to prepare erythrocyte ghosts which either are semipermeable to potassium or have become fully permeable or leaky. Experimentally, this was done by lysing them in the presence of either water, EDTA, or a variety of divalent cations and then resealing them by raising the salt concentration to 0.17

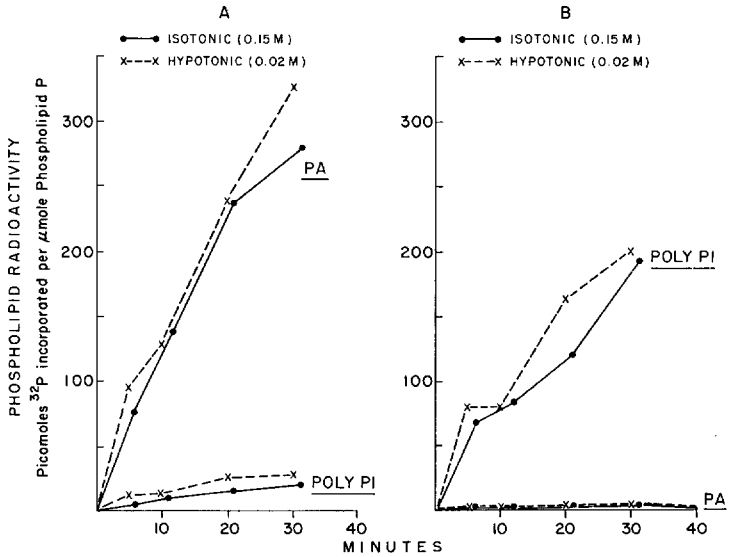


FIGURE 5 Effect of tonicity and calcium on the incorporation of γ-AT^{32}P into phospholipids of erythrocyte membranes prepared in the presence of 1 mM EDTA. EDTA-lysed erythrocyte membranes were prepared as described in Materials and Methods. They were incubated in Krebs-Ringer phosphate and 0.1 μmole γ-AT^{32}P/ml (5×10^7 cpm/μmole) in the presence or absence of calcium and with either isotonic NaCl (0.15 M) or a hypotonic saline solution (0.02 M). Experiment A was carried out in the presence of 2.58 mM calcium. In experiment B, no calcium was added.

M with a 5:1, KCl:NaCl, solution. The resealed cells entrap potassium when they are incubated at 37°C for 40 min. The cells were then washed to remove extracellular potassium, and the amount of potassium retained within the cell was used as a measure of the intactness of the reconstituted membrane. Reconstituted membranes so prepared were incubated with γ-AT^{32}P, and the incorporation of ^{32}P into phospholipids was measured and compared with the ability of the reconstituted cell to retain potassium.

The ability of cells, lysed in different calcium concentrations, to retain potassium is shown in Fig. 6. As the calcium level of the lysing solution is raised, the membranes fail to reseal and they lose their intracellular potassium. Maximum loss of potassium occurs when cells are lysed in 0.5 mM calcium. The incorporation of γ-AT^{32}P into phospholipid was low in cells prepared in 0.25 and 0.5 mM calcium, but it increased in cells prepared with higher concentrations of calcium. There is some relation between loss of potassium from the cell and increased phospholipid metabolism. This relation is not, however, a strict one. For instance, at 0.5 mM Ca^{++} the cells had completely lost their ability to retain potassium but there was little or no increase in phospholipid metabolism (Fig. 6).

Other divalent cations added to the lysate were also found to have an influence on the reconstitution properties of the membranes. Barium and strontium acted like magnesium, in that they allowed the cell to regain its semipermeable properties to potassium. Other divalent cations such as zinc, calcium, lead, and mercury acted like calcium in affecting the membrane so that it would not reconstitute. If the cells were lysed in water or in EDTA, they also failed to retain potassium (Table I).

Ghosts, prepared by lysis with different cations, were incubated with γ-AT^{32}P. Those ghosts which were able to retain potassium (lysed in Mg^{++}, Ba^{++}, or Sr^{++}) had the lowest incorporation into the phospholipids. Ghosts which were leaky to potassium had a higher incorporation. There was not, however, a quantitative relation between the amount of potassium lost and the increase in phospholipid metabolism. Ghosts prepared in the presence of mercury or lead lost as much potassium as those prepared with cadmium or zinc, but they incorporated less phosphate into phosphatidic acid and the polyphosphoinositides (Table IV, experiment 3). In general, however, those cells which failed to retain potassium had an increased phospholipid metabolism.

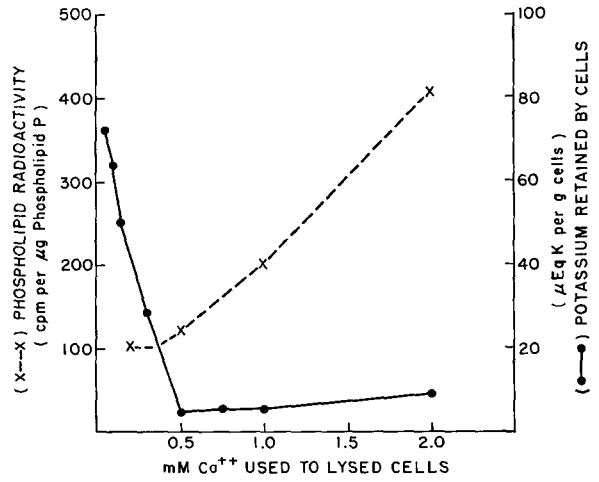


FIGURE 6 Effect of calcium on the reconstitution of erythrocyte ghosts and on phospholipid metabolism. Washed cells were lysed in 10 volumes of a calcium solution with different concentrations as noted. The lysed membranes were then reconstituted by adding a salt solution to a final concentration of 0.17 M. The salt solution was 3/1, K/Na. The cells were incubated at 37°C for 40 min, and then were washed three times in 0.17 M NaCl in 0.01 M Tris, pH 7.4, to remove the extracellular potassium. Some of the washed cells were kept to determine the amounts of retained potassium as measured by flame photometry, and others were incubated with γ-AT^{32}P (0.1 μmole/ml) in isotonic Krebs-Ringer phosphate.

TABLE I

The Effect of Various Cations on the Ability of the Erythrocyte Ghost to Retain Potassium and to Incorporate $\gamma AT^{32}P$ into Phospholipids

Experiment	Cation present during lysis	K^+ retained	Radioactivity $CHCl_3$ extract	Phospholipid radioactivity	
				PA	Poly PI
		μEq/g cell	*cpm/μg phospholipid*	*cpm/μg phospholipid*	
1	Magnesium	48	172	46	35
	Calcium	10	300	174	52
	(water)	18	692	28	294
	(EDTA)	15	412	40	94
2	Magnesium	40	180	50	6
	Strontium	36	192	23	52
	(water)	15	639	16	361
	Zinc	8	1832	107	1012
3	Magnesium	40	28	—	—
	Barium	51	40	3	7
	Strontium	36	54	18	18
	Lead	5	226	12	52
	Mercury	5	114	—	24
	Zinc	8	1760	56	540
	Cadmium	9	2040	—	926

The cells were lysed in either 10 volumes of water or 1 mM of the cation, or EDTA. The cells were resealed with 0.17 M salt containing 5/1 K^+/Na^+, and were then incubated at 37°C for 40 min. The resealed cells were washed three times with 0.01 M Tris, pH 7.4, with 0.17 M NaCl. The amount of K^+ retained in the cell was determined by flame photometry. The K^+ values are the averages of two experiments. Normal red cells contain about 90 μEq of K^+/g cells. The resealed cells were also incubated with γ-$AT^{32}P$ as described in Fig. 2.

The $CHCl_3$ extract represents the total phospholipid radioactivity. *PA*, phosphatidic acid; *Poly PI*, polyphosphoinositides.

The ghosts prepared in magnesium or calcium had a greater percentage of the incorporated radioactivity in phosphatidic acid, whereas ghosts prepared in water, EDTA, and the other divalent cations incorporated ^{32}P mostly into triphosphoinositide.

Phospholipid Metabolism in Intact and Lysed Red Cells and Measurement of the Intracellular Pool of ATP

The intact erythrocyte membrane is impermeable to ATP. In lysed cells, however, the ATP may enter the cell. If the incorporation of γ-$AT^{32}P$ into the phospholipids occurs only on the inside face of the vesicular membrane, then the increased phospholipid metabolism of open cells may merely be due to a larger entry of γ-$AT^{32}P$ into the vesicle. To determine whether this was responsible for the increased phospholipid metabolism, attempts were made to load the erythrocyte with γ-$AT^{32}P$ by lysing the cells in the presence of γ-$AT^{32}P$ and then resealing the vesicle with the radioactive precursor inside. This could be accomplished, but the amount of γ-$AT^{32}P$ entrapped in the vesicle was too small and too easily hydrolyzed to permit measurement of lipid metabolism. Therefore, to determine whether changes in the intracellular γ-$AT^{32}P$ levels are responsible for the phospholipid effect, experiments were performed with intact red cells, incubated in a medium containing inorganic ^{32}P. These cells will allow the entry of inorganic ^{32}P and will convert part of it, by glycolysis, into γ-$AT^{32}P$. As a measure of the intracellular γ-$AT^{32}P$ pool, the specific activity of the nucleotide phosphates within the cell was determined. Cells were incubated in media containing different amounts of either NaCl or sucrose. Also meas-

TABLE II

Lysis of the Erythrocyte and the Incorporation of ^{32}P into Nucleotide Phosphates and Phosphatidic Acid

Conditions of incubation	Hb lost	Radioactivity		
		Nucleotide P	Phosphatidic acid	PA × 10^5 / nucleotide P
		cpm/mg P	*cpm/mg phospholipid P*	
1. 0.05 M Tris, pH 7.4, 10^{-4} NaF 5 mM $MgCl_2$				
a. NaCl 0.0125 M	90	1.6×10^5	134	84.0
b. NaCl 0.0625 M	70	1.9×10^5	170	89.5
c. NaCl 0.16 M	0	0.64×10^5	11	17.2*
2. 0.01 M Tris, pH 7.4, 5 mM $MgCl_2$				
a. 0.01 M sucrose	100	2.2×10^4	28	127.0
b. 0.03 M sucrose	90	2.1×10^4	41	195.5
c. 0.07 M sucrose	50	2.0×10^4	42	210.0
d. 0.27 M sucrose	4	3.8×10^4	42	58.0*
3. Krebs-bicarbonate buffer				
a. No NaCl	90	2.8×10^5	389	139.0
b. With NaCl (0.15 M)	4	2.0×10^5	53	26.0*

Washed red cells were incubated with inorganic ^{32}P in the incubation media described above. Incubations were carried out at 37°C for 20 min. Experiments 1 and 2 utilized 6 μCi ^{32}P/ml of incubation medium, and experiment 3 utilized 10 μCi of ^{32}P per ml.

* Conditions under which the red cell is not lysed. The hemoglobin lost was determined spectrophotometrically. The ratio is the specific activity of the phospholipid divided by that of the nucleotide phosphates × 10^5. The specific activity of nucleotide phosphates was determined by adsorption on charcoal and hydrolysis in 1 N acid for 10 min at 100°C, as described by Crane and Lipmann (26). *PA*, phosphatidic acid.

ured were the radioactivity of the phospholipids and the amount of hemoglobin lost.

Lysis of the red cell by hypotonic solutions caused changes in the specific activity of the nucleotide phosphates, and these changes differed depending on the medium in which the cells were incubated and also on the material used to change the tonicity of the medium. Lysis of the red cell by decreasing the NaCl concentration caused an increase in the specific activity of the intracellular nucleotide phosphates. This increase in specific activity when the cell was lysed was more pronounced when the cells were incubated in Tris buffer, pH 7.4, than when incubated in Krebs-bicarbonate buffer. In contrast to the above, lysis of red cells with decreasing sucrose concentrations caused a decrease in specific activity of the nucleotide phosphates (Table II). The radioactivity in phosphatidic acid was increased when the cells were lysed. Maximum phospholipid incorporation was noticed as soon as hemoglobin was lost from the cell. Complete lysis was not necessary to obtain this effect. The increased phospholipid effect was less noticeable when cells were incubated with sucrose (Table II). There was little labeling of phosphatidic acid when the cell remained intact, although the intracellular nucleotide phosphates were labeled.

To correct for differences in the entry of inorganic ^{32}P, or for differences in rates of glycolysis in the cell, the radioactivity found in phosphatidic acid was corrected to a constant specific activity of the intracellular nucleotide phosphates. The values are shown in the last column of Table II. In all instances, regardless of the mode of lysis of cells or of the changes in the intracellular ATP pool, the corrected radioactivity in phos-

phatidic acid was always higher in open cells than in intact cells.

Entry of γ-AT^{32}P into Reconstituted Ghosts Prepared in the Presence of Various Cations

The previous experiments showed that the intracellular ATP pool can change with lysis but that the changes in intracellular ATP cannot account for the phospholipid effect noticed. Those experiments were done by incubating with inorganic ^{32}P. However, most of the studies were performed with γ-AT^{32}P in the incubating medium, and hence it is important to determine directly the amounts of γ-AT^{32}P that entered the cells from the medium. As mentioned before, an attempt to entrap γ-AT^{32}P into reconstituted ghosts and then to measure phospholipid metabolism was unsuccessful. An estimate of the amount of γ-AT^{32}P which entered the cell could be obtained, however, by incubating intact ghosts (lysed with Mg^{++}) and leaky ghosts (lysed with Ca^{++}, Cd^{++}, or water) with γ-AT^{32}P, and by recovering the cells by centrifugation at 2°C for 5 min at 10,000 *g*. The amount of γ-AT^{32}P in the pelleted cells was then determined, as well as the radioactivity in the extracted phospholipids. The cells were added to a buffered saline medium with γ-AT^{32}P which was kept at 0°C. The zero time measurement was taken by immediately centrifuging after the addition of the cells. In Table III it is seen that at zero time of incubation there was little difference in the levels of γ-AT^{32}P found within the cell. During incubation at 37°C the γ-AT^{32}P within the cell was utilized and the levels dropped. Some cells used more ATP than others. For instance, the cells prepared by lysis in cadmium used less ATP in 30 min than did the other cells. The greater incorporation of ^{32}P into the phospholipids of leaky cells cannot, however, be ascribed to a greater entry of γ-AT^{32}P into those cells, since the intracellular levels of γ-AT^{32}P were similar but the phospholipid radioactivity was greatly enhanced in leaky cells (Table III). The cells lysed in magnesium were intact while the others were leaky to potassium.

Treatment of Intact Cells with Digitonin and Primaquine Phosphate

Intact cells were incubated in Krebs-Ringer bicarbonate, with ^{32}P, in the presence and in the absence of digitonin. Digitonin is known to remove cholesterol from the membrane and thus cause lysis (29). Digitonin had no effect on the incorporation of ^{32}P into phospholipids, although incorporation into the nucleotide phosphate pool was slightly enhanced. This experiment again shows that an increased radioactivity in the nucleotide phosphate pool is not necessarily reflected by an increased phospholipid metabolism (Table IV).

Primaquine phosphate, which produces lysis and resealing into small vesicles (28), caused a decrease in the specific activity of the nucleotide phosphates and an inhibition of the incorporation of ^{32}P into the phospholipids (Table IV).

TABLE III

Uptake of γ-AT^{32}P by Cells Lysed under Different Conditions

Preparation of ghosts	γ-AT^{32}P levels in the cell			Incorporation of γ-AT^{32} P into phospholipids		
	0 min	15 min	30 min	0 min	15 min	30 min
	cpm/μg phospholipid P			*cpm/μg phospholipid P*		
Water	32500	8150	6050	620	1660	3720
Magnesium	23000	19500	6100	50	100	50
Calcium	22500	4850	5500	30	270	390
Cadmium	38200	18500	13000	600	2380	3600

The cells were lysed in either water or 1 mM of the cation as described in the text. The cells were washed thoroughly with 0.17 M NaCl in 0.01 M Tris, pH 7.4, and then incubated at 37°C in 0.15 M NaCl, 0.01 M, Tris, pH 7.4, with 0.2 μmoles of γ-AT^{32}P per ml (3.9×10^5 cpm/μmole). At the desired time the reaction was stopped by centrifuging the cells at 10,000 *g* for 5 min at 2°C and by adding cold 5% TCA to the pellet. The γ-AT^{32}P level is the radioactivity absorbed on charcoal from the TCA-soluble portion which is hydrolysed in 1 N acid at 100°C after 10 min. All values are corrected to a constant amount of phospholipid P.

TABLE IV

Effect of Primaquine and Digitonin on the Incorporation of ^{32}P into the Nucleotide Phosphates and Phospholipids of Intact Red Cells

Experiment	Addition	Nucleotide P	Phospholipids	Ratio × 10^5
		cpm/mg P	*cpm/mg P*	
1	None	3.6×10^4	385	10.7
	Primaquine, 3.6×10^{-3} M	2.2×10^4	165	7.5
	Primaquine, 1.6×10^{-2} M	2.0×10^4	145	7.5
2	None 15 min at 37°C	1.5×10^4	80	5.3
	Digitonin, 18 min at 37°C	1.6×10^4	80	5.0
	None, 45 min at 37°C	1.5×10^4	140	9.2
	Digitonin, 48 min at 37°C	1.7×10^4	160	9.3

Whole washed red cells were incubated with ^{32}P (1 μCi/ml) in Krebs-Ringer phosphate at 37°C. In experiment 1 the incubation was carried out for 60 min. In experiment 2 the incubation was as described above. The concentration of digitonin was 3.2×10^{-4} M.

The specific activity of the nucleotide phosphates was determined as described in the text. The ratio is the radioactivity of the phospholipids divided by the specific activity of the nucleotide phosphates.

TABLE V

Effect of CTP and Calcium on the Incorporation of γ-AT^{32}P and Inositol-^3H into Membrane Phospholipids

Experiment	Radioactive substrate	Additions	Radioactivity into phospholipids			
			No salt		Salt	
			PA	Poly PI	PA	Poly PI
			cpm/μg P			
1	γ-AT^{32}P	None	2850	435	1130	284
		CTP	1760	307	883	191
		Inositol	2385	488	968	289
		CTP + Inositol	1360	232	780	172
2	γ-AT^{32}P	None	346	67	186	28
		Ca^{++}	640	78	190	38
		Ca^{++}, CTP	242	36	182	34
			Total Phospholipids		Total Phospholipids	
3	Inositol-^3H	None	358		105	
		Ca^{++}	28		32	
		CTP	6830		7000	
		CTP + Ca^{++}	137		108	

Membranes were incubated in Krebs-Ringer phosphate with or without calcium. Sodium chloride was added to some at a final concentration of 0.15 M.

Experiments 1 and 2 were performed with ghosts prepared by lysis in 1 mM $MgCl_2$ as described in the text. The AT^{32}P was 5×10^5 cpm/mole for experiment 1, and 10^5 cpm/μmole for experiment 2. In both cases the incubation medium contained 0.28 μmoles/ml of ATP. Incubation was carried out for 45 min at 37°C.

Experiment 3 was performed with ghosts prepared by lysis in 1 mM EDTA. The inositol-2-^3H used was 1 μCi/ml of incubation mixture, with a specific activity of 3.4 mCi/μmole. *PA*, phosphatidic acid; *poly PI*, polyphosphoinositides. The calcium and CTP concentrations were 5×10^{-4} M.

Effect of CTP and Inositol on the Incorporation of ^{32}P from γ-AT^{32}P into Membrane Phospholipids

Cytidine triphosphate (CTP), although a necessary cofactor for synthesis of the phosphoinositides from phosphatidic acid, did not enhance the incorporation of γ-AT^{32}P into the polyphosphoinositides, but actually inhibited the incorporation into both phosphatidic acid and polyphosphoinositides. The presence of inositol had no effect. Earlier studies by Hokin and Hokin showed that CTP inhibited the incorporation of γ-AT^{32}P into di- and triphosphoinositide, and had little effect on phosphatidic acid (5). As seen in previous experiments, incubation of the membranes in hypotonic medium caused an increased incorporation into the phospholipids (Table V). Calcium, added by itself, stimulated the incorporation of ^{32}P into phosphatidic acid (Table V, experiment 2). In EDTA-lysed cells the increased incorporation into phosphatidic acid caused by added Ca^{++} was followed by a decrease in the incorporation into polyphosphoinositides (Fig. 5).

CTP, however, greatly enhanced the incorporation of inositol-^{3}H into the phospholipids of cells prepared by lysis in EDTA. Salt had no consistent effect on the incorporation. Calcium severely inhibited the incorporation in the presence or absence of CTP. Inositol-^{3}H was incorporated into a phospholipid with the same chromatographic mobility as phosphatidylinositol. There was no detectable incorporation into the polyphosphoinositides.

Other Phospholipid Metabolic Pathways in the Erythrocyte Membrane

The erythrocyte is capable of incorporating free fatty acids into the phospholipids. When palmitic acid-^{14}C was absorbed to albumin and incubated with washed, intact erythrocytes, rapid incorporation into the triglycerides and the phospholipids, but mostly into the triglycerides, was noted. The cells which were incubated in hypotonic medium were slightly less active in incorporating palmitate into triglycerides than were the cells incubated in complete isotonic Krebs-Ringer. The incorporation of palmitate into triglyceride leveled at 5 min of incubation in the isotonic medium but fell slightly in the hypotonic medium. The majority of the radioactivity in the phospholipid was in phosphatidylcholine and phosphatidylethanolamine. With cells incubated in hypotonic media, it was difficult to measure the incorporation into phosphatidylethanolamine because of the appearance of a material that streaked in the chromatographic systems which were used. There was a small increased incorporation of palmitate into phosphatidylcholine in cells incubated in isotonic saline (Fig. 7).

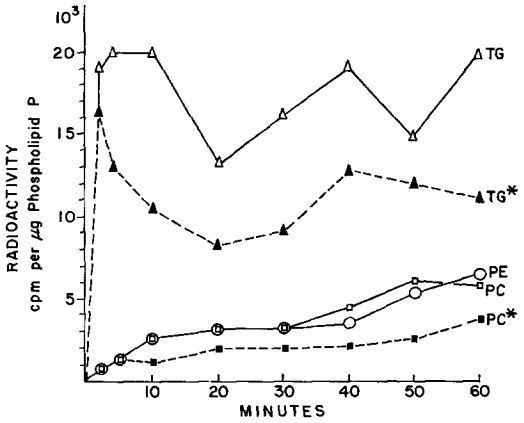


FIGURE 7 Incorporation of ^{14}C-fatty acids into the lipids of intact and lysed erythrocytes. Washed red cells were incubated in Krebs-Ringer phosphate with or without salt. Palmitate-^{14}C adsorbed to human albumin, prepared as described in the text, was added to the incubation mixture. The medium contained 100,000 cpm of palmitate-^{14}C/ml. Incubation was done at 37°C. The radioactivity in the lipids was determined as described in Materials and Methods. *TG*, triglyceride; *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine. The asterisks denote incubation in hypotonic Krebs-Ringer phosphate.

The incorporation of α-glycerophosphate-^{32}P into the phospholipids was also measured. α-glycero-^{32}P (2×10^7 cpm/μmole) was incubated with red cells in the presence of coenzyme A (CoA), ATP, and added fatty acids, or with stearyl and palmityl CoA. No incorporation into the phospholipids was detected in either the presence or absence of saline.

In order to determine whether the incorporation of γ-AT^{32}P into the phospholipids could be due to an exchange of phosphate of the phospholipids with inorganic phosphate in the media, erythrocyte membranes were incubated with γ-AT^{32}P in hypotonic media for 1 hr as described in Fig. 2. The isolated membranes were then washed thoroughly with nonradioactive medium and reincubated at 37°C for 30 min

in 0.05 M phosphate buffer in the absence of ATP. There was no change in radioactivity of the membrane phospholipids during the second incubation, showing that exchange with inorganic phosphate nad not occurred.

DISCUSSION

The lipid composition of the human erythrocyte membranes has been well documented. The membranes can be obtained in good yield without losing their lipid components (18), and they can be reconstituted insofar as they can regain their semipermeable properties to sodium and potassium (8, 17). Although the erythrocyte membrane is not actively synthesizing all of its lipids *de novo*, it is capable of selective synthesis of certain lipids and lipid moieties (6). The property of the erythrocyte membrane to reseal after lysis and the low metabolic background of its phospholipids make it valuable as a model with which to study the chemical reactions which a membrane may be undergoing during damage and repair. That phospholipids may be involved in such repair mechanisms of the membranes is suggested by work from many different laboratories. For example, there is a relation between increased turnover of certain phospholipids, mainly phosphatidic acid and phosphatidylinositol, and secretion (19, 20). Although some of this effect may be due to other processes, such as the hormonal action which triggers secretion, or to other aspects of secretion, part of it may be due to the pinching off and fusion of membranes which is thought to accompany the intracellular transport of proteins. There are other findings which suggest that phospholipid metabolism may accompany membrane rupture and resealing. Leukocytes involved in phagocytosis have an increased metabolism of certain lipids (21, 22), as do HeLa cells treated with digitonin (23), and leukocytes caused to agglutinate by the addition of phytohemagglutinin (24). An example, that increased phospholipid metabolism of the erythrocyte membrane may accompany membrane damage, has been given previously by Jacob and Karnovsky (27). They have shown that red cells suspended in a hypotonic medium that did not cause hemolysis incorporated more inorganic ^{32}P into a "phosphatidylserine" fraction than did cells suspended in isotonic medium. They also showed that red cells from a patient with hereditary spherocytosis incorporated more phosphate into the phospholipids than did normal cells when both were incubated in identical isotonic medium. These different systems all have in common the fact that the membranes either are damaged or are undergoing breakage and resealing.

The difference between the amounts of incorporation of γ-AT^{32}P into the lipids of erythrocyte ghosts incubated in hypotonic or in isotonic medium is pronounced. This difference does not depend on the type of salt used to change the tonicity of the medium, since the lack of either sodium chloride, phosphate buffer, or sucrose will cause increased metabolic activity (Figs. 2 and 4, and Table I). This increased metabolism does not depend on tonicity, since tonicity has no effect on ghosts which have been lysed in the presence of EDTA (Fig. 5). Also, ghosts prepared by lysis with equal concentrations of different divalent cations incorporate different amounts of γ-AT^{32}P into the phospholipids, although they were subsequently incubated under the same conditions of tonicity (Table IV). These results demonstrate that ghosts or cells which are open in that they either release hemoglobin or fail to retain potassium, incorporate more γ-AT^{32}P into the phospholipids than do those cells which are closed. This may be most easily explained if open cells allowed the entry of more substrate than did closed cells, and is of special significance because intact erythrocytes are relatively impermeable to ATP. Two types of experiments were performed to determine whether this could explain the increased incorporation found to occur in open cells. One set of experiments measured the specific activity of the intracellular pool of nucleotide phosphates when open and closed cells were incubated with inorganic ^{32}P, and the other experiments measured the actual amount of γ-AT^{32}P which entered resealed and leaky ghosts (Tables II and III). Both of these experiments indicated that the increased phospholipid metabolism noted in leaky cells could not be accounted for by differences in the intracellular level of γ-AT^{32}P (Tables II and III).

As a working hypothesis it is postulated that increased phospholipid metabolism is associated with a repair mechanism of the membrane, and that osmotic lysis or leakiness, which may result from deleting or exchanging certain divalent cations, occurs by the opening of pores at certain regions along the membrane. It is envisioned

that lipoproteins are situated at these porous regions and that they contain phospholipids such as phosphatidic acid and the polyphosphoinositides. The opening and resealing of the pores on the membrane may involve a phosphorylation and dephosphorylation of the lipid moieties of these lipoproteins.

This scheme takes into account observations made in this study. When the membrane is open due to either osmotic lysis or treatment with EDTA, calcium, strontium, or cadmium, there is an increased incorporation of ^{32}P of γ-AT^{32}P into the phospholipids. Other phospholipid metabolic reactions such as the acylation of lipids (Fig. 6) and the incorporation of inositol-^{3}H (Table V) are not stimulated by increased leakiness. α-Glycerophosphate is not incorporated into phospholipids, and there is no acylation of phosphatidic acid and the polyphosphoinositides. Hence, the reaction which is stimulated is a specific one, the phosphorylation of lipid moieties leading to the formation of phosphatidic acid and polyphosphoinositide. Virtually all of the radioactivity in the polyphosphoinositides is monoesterified; this coupled with the lack of incorporation of α-glycerophosphate and fatty acids into this lipid shows that there is little *de novo* synthesis but mainly a turnover of the phosphate moieties.

This hypothesis predicts that there are certain regions in the membrane which are more susceptible to lysis and which contain the functional lipoproteins involved in the opening and closing of "pores." This would explain why added digitonin does not stimulate these enzymes. Digitonin removes cholesterol (29), and this may occur at a different location on the membrane. Primaquine phosphate ruptures and reseals the membrane to give smaller vesicles (28). Since the membrane is vesicularized or resealed by primaquine phosphate and does not remain open, increased phospholipid metabolism is not expected, since this only becomes apparent when the membrane is open. The incorporation ceases when the membrane reseals.

The technical assistance of Mr. P. Y. Liu is gratefully acknowledged. Thanks are due to Professor Abraham Mazur of the New York Blood Center and the City University of New York, and to Dr. Mabel Hokin of the University of Wisconsin for many helpful suggestions during the preparation of the manuscript; and also to Dr. John Derrick of the New York Blood Center for performing the sodium and potassium determinations.

This work was supported by Grants AM13620 from the National Institute of Arthritis and Metabolic Diseases, and HE09011 from the National Heart and Lung Institute.

Received for publication 7 July 1970, and in revised form 17 August 1970.

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