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In vitro Incorporation of Radiophosphorus into the Phosphatides of Normal Human Blood Cells.* (29830)

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In the present study, normal human blood was incubated with P^{32} labeled orthophosphate (P^{32}), the cells separated, and quantitative measurements of P^{32} incorporation into various phosphatides of red cells, leukocytes and platelets were made. Previous reports of measurements of the turnover of the phospholipid, phosphatidic acid, in the red cell have not shown consistent results(1-4). Similarly, the *in vitro* activity of leukocyte and platelet phosphatides, as measured by P^{32} uptake, differs in normal cells separated prior to incubation(5,6), in leukemic WBC(7) and in WBC obtained by peritoneal irritation(8). Since phosphatide activity in normal circulating whole blood cells, not separated prior to incubation, might also vary, this could partially explain the inconsistency of results. Values for normal cells similarly prepared would be useful for comparison to leukemic WBC and those obtained by peritoneal stimulation.

Methods. Fresh human blood anticoagulated with heparin or Na_2 EDTA was mixed with a solution containing 15 μc of high specific activity $Na_2HP^{32}O_4$ (or 30 μc acetate-1-C^{14}) and 1 mg glucose per ml packed RBC. The pH was maintained at 7.30 $\pm$ 0.10 by adjusting the flow of 5% CO_2 in air through the mixture. After constant shaking at 37°C for the desired time, aliquots were removed and erythrocyte, leukocyte or platelet concentrates prepared. Samples were obtained at regular intervals in studies in which the rate of incorporation of P^{32} was being determined and at 3.2 hours for all studies in which uptake into the various cell types was being compared. To obtain *RBC fractions,* samples of whole blood were thrice centrifuged and washed with 0.9% NaCl with removal of the buffy coat after each centrifugation. Less than 1 WBC and 2 platelets per 5×10^3 RBC were observed. *Leukocytes* were obtained by sedimentation of whole blood with 3% dextran (Mol. Wt. 200,000-275,000, Sigma) in 0.9% NaCl at 4°C. The sediment was washed thrice with 0.9% NaCl. These fractions contained cell ratios which ranged between 3-16 RBC/20-25 platelets/1 WBC. Calculations were corrected for contaminating red cells and platelets. *Platelets* were obtained by differential centrifugation. The platelet rich sediment was washed 3 times with 0.9% NaCl. Leukocytes and red cells were not seen during the platelet counts. Erythrocyte and WBC counts were performed by use of an electronic cell counter, and platelet counts were done with phase microscopy. Additional counts and observations were made on blood films after staining with Wright's stain.

Extraction of the lipids of all cell types was accomplished by previously described techniques(9). Phosphorus was determined by the method of Bartlett(10). Column chromatographic separation of phospholipids was performed as modified by Chang and Swee-

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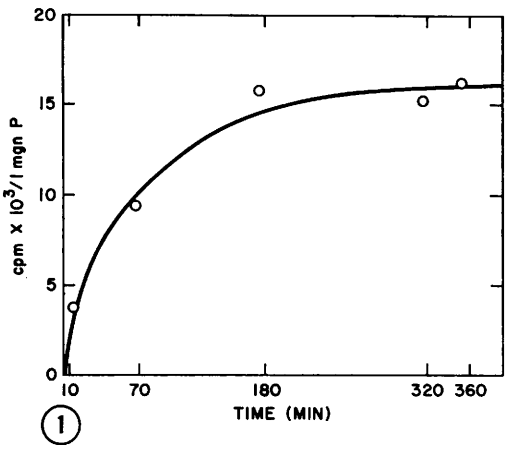
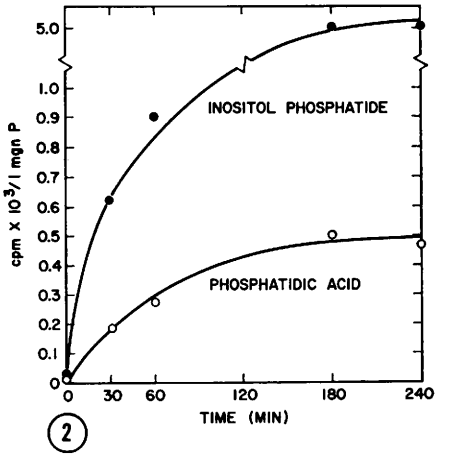
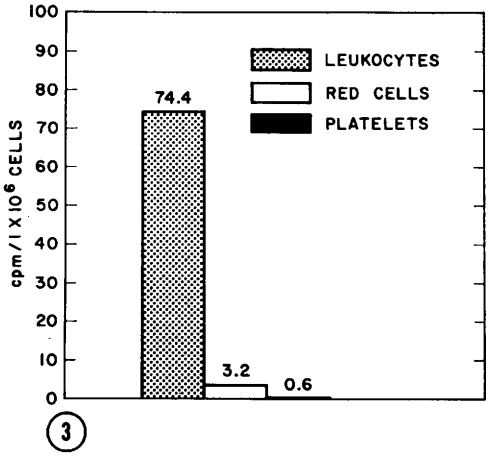
ley(11), and paper chromatography was performed on silicic acid-impregnated paper according to methods modified for the red cell by Reed(12). The separate phosphatides were eluated from the silicated paper with 0.1 N HCl in redistilled methanol and the phosphorus content of the eluate determined. Thin layer chromatography was performed with Silica Gel G using a chloroform : methanol : water (50:20:3 V/V) solvent system (13). The R_F values which were obtained for the phosphatides on paper and thin layer chromatography were similar to those previously observed(14,12,7,13,9). The R_F value for phosphatidic acid on the thin layer chromatograms was similar to that described by Rouser(15). Identification was further obtained by use of known compounds as markers and by the use of Rhodamine-6-G, ninhydrin, the periodic acid Schiff stain and by iodine vaporization.

Radiolabeled phosphatide activity was determined by cutting the paper chromatogram into sections or by transferring the identified







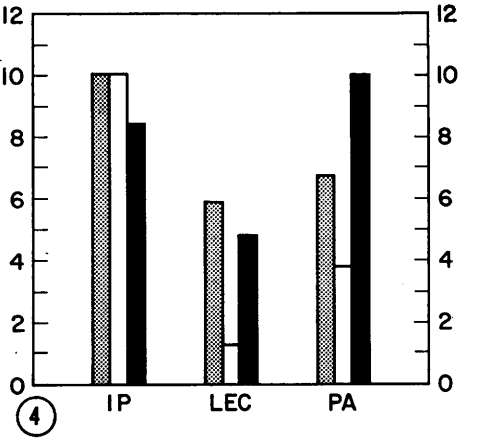


FIG. 1. Incorporation of P^{32} labeled orthophosphate into red cell phosphatidic acid. Whole blood sample incubated as described in text with 25 μc $Na_2HP^{32}O_4$ per 1 ml red cells.

FIG. 2. Incorporation of P^{32} labeled orthophosphate into various platelet phosphatides. Whole blood sample incubated as described in text with 7 μc $Na_2HP^{32}O_4$ per 1 ml red cells.

FIG. 3. *In vitro* incorporation of P^{32} labeled orthophosphate into phosphatidic acid of various formed elements of normal human blood.

FIG. 4. Relative specific activities of leukocyte phosphatides as estimated by various authors. Results have been compared by assigning the value 10 to the phosphatide with the highest specific activity in each study. Shaded, Karnovsky and Wallach; □ Firkin and Williams; ■ Present study. IP = Inositol Phosphatide, LEC = Lecithin, PA = Phosphatidic Acid.

TABLE I. Radioactivity* of Various Phosphatides Per Cell in Formed Elements of Normal Human Blood After *in vitro* Incubation with Radiophosphorus.

		Phosphatidic acid	Lecithin	Inositol phosphatide
Erythrocyte†	Mean	3.21 ± .22‖		
	Range	1.70 – 4.80		
Platelet‡	Mean	.60 ± .06	3.17 ± .34	7.89 ± .74
	Range	.30 – .90	1.50 – 4.70	5.5 – 13.8
Leukocyte§	Mean	74.4 ± 7.3	205.3 ± 20.5	63.7 ± 13.7
	Range	44 – 98	97 – 276	27 – 115

* CPM/1 × 10^6 cells.
† 21 studies.
‡ 10 "
§ 8 studies.
‖ Standard error.

TABLE II. Specific Activity* of Phosphatides of Formed Elements in Normal Human Whole Blood After *in vitro* Incubation with Radiophosphorus.

		Phosphatidic acid	Lecithin	Inositol phosphatide
Erythrocyte†	Mean	12.0 ± .8‖		
	Range	6.2 – 17.1		
Platelet‡	Mean	1.49 ± .15	1.25 ± .17	23.1 ± 2.8
	Range	.69 – 2.04	.69 – 1.89	12.4 – 34.9
Leukocyte§	Mean	6.76 ± .44	2.55 ± .44	5.67 ± .91
	Range	5.09 – 8.00	1.60 – 4.76	3.27 – 9.82

* CPM × 10^3/1 mg phosphorus.
† 21 studies.
‡ 10 "
§ 8 studies.
‖ Standard error.

phosphatide from the thin layer plate to planchets and counting the radioactivity in a gas flow detector.

Results. The incorporation of P^{32} into the phosphatides of the various types of cell-rich fractions demonstrated a maximal incorporation within 3 hours following which a plateau was maintained for at least an additional 6 hours. Representative uptake curves of red cell and of platelet phosphatides are shown in Fig. 1, 2.

P^{32} incorporation into erythrocyte phosphatides. The RBC were unique in that radioactivity was found only in phosphatidic acid (Table I, II). Other phosphatides (lecithin, sphingomyelin, phosphatidyl serine, phosphatidyl ethanolamine and inositol phosphatide) had negligible or no radioactivity. Incubation of similarly prepared RBC with acetate-1-C^{14} on 2 separate occasions showed no incorporation of acetate-1-C^{14} into the phosphatides including phosphatidic acid.

P^{32} incorporation into platelet phosphatides. Ten samples were analyzed. Peak radioactivity was present in the inositol phosphatide, lecithin and phosphatidic acid fractions. Incorporation of P^{32} into the inositol phosphatide fraction per 1 × 10^6 platelets was greatest (Table I). Of the other phospholipids, the highest degrees of incorporation per 1 × 10^6 platelet were in the lecithin fraction (3.17 CPM/1 × 10^6 platelets). The specific activity of platelet inositol phosphatide (23.1 CPM × 10^3/1 mg P) (Table II) was similarly highest and was approximately 15 times that noted in phosphatidic acid (1.49 CPM × 10^3/1 mg P).

P^{32} incorporation into leukocyte phosphatides. Peak radioactivity was observed in the inositol phosphatide, lecithin and phosphatidic acid moieties. P^{32} incorporation was greatest in the lecithin moiety per 1 × 10^6 WBC and showed less incorporation into the phosphatidic acid and inositol phosphatide fractions as related to 1 × 10^6 WBC (Table I). In terms of specific activity, phosphatidic acid and inositol phosphatide were greatest (Table II).

Discussion. The incorporation of P^{32} into normal RBC phosphatides is limited to phosphatidic acid but also occurs in other phosphatides of leukocytes and platelets. P^{32} incorporation into phosphatidic acid is approximately 23 times greater per WBC than per

RBC and nearly 125 times greater per WBC than per platelet (Fig. 3). When the RBC/WBC ratio in whole blood is normal (approximately 1000:1), 2 to 4% of the P^{32} incorporation into the total phosphatidic acid would be expected to be contributed by white cells. When the normal RBC : platelet ratio (20:1) is present, the expected contribution of platelet phosphatidic acid radioactivity to whole blood is less than 1%. Thus, most of the P^{32} activity of phosphatidic acid in normal whole blood can be attributed to the numerically predominant erythrocyte. Other phosphatides, however, which are in abundance in white cells and platelets, may contribute significantly to the phosphatide activity of whole blood samples despite the relatively small number of leukocytes and platelets in normal whole blood. For example, inositol phosphatide activity which is high per single WBC and platelet was not detected in RBC-rich samples. In cell suspensions with greater WBC and platelet concentrations, P^{32} incorporation into inositol phosphatide would be more readily evident. For precise analyses of the P^{32} activity of phosphatidic acid and other phosphatides in whole blood, it would be necessary to determine the contribution of the leukocytes and platelets which are present. Differences in cell concentrations may partially explain the qualitative and quantitative variations found in the activity of RBC phosphatides by different observers(1-4).

Firkin and Williams(7) measured the degree of *in vitro* incorporation of P^{32} into the phospholipids of leukemic leukocytes. The highest specific activities in leukocytes of a patient with chronic myelocytic leukemia were found in phosphoinosotide and phosphatidic acid. Similar examinations were made by Karnovsky and Wallach(8) with "resting" WBC obtained from peritoneal exudates of guinea pigs. They found the highest specific activity in inositol phosphatide and lesser but distinct amounts in phosphatidyl serine, phosphatidic acid and lecithin. A comparison of the findings between the studies is shown in Fig. 4. The results of the present study are similar to those of Karnovsky and Wallach(8). The variation among the 3 studies may be due to differences in phospholipid metabolism between circulating leukemic leukocytes, "resting" white cells and normal circulating white cells. Whether or not the differences in relative specific activity of phosphatidic acid in the leukemic white blood cell and the normal white cell are associated with differences in membrane transport as described for other cells(16) cannot be stated.

Buchanan(17) and Marks *et al*(18) using acetate-1-C^{14} have shown that negligible synthesis of lipid occurs in normal mature human erythrocytes. Their observations were confirmed in the present study and extended to show that synthesis in all phosphatides of the normal RBC was negligible with no evidence of localization to a single phospholipid.

Summary. The incorporation of P^{32} into the phosphatides of the formed elements of normal human blood has been measured. A characteristic pattern was described for erythrocytes, leukocytes and platelets. Radiophosphorus is incorporated into red cell phosphatidic acid. The variable and high rates of incorporation of P^{32} into certain phosphatides of red cells, white cells and platelets would indicate the necessity of assessing this function by these cells when making such measurements in whole blood.

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Influence of Electrolytes on Stability of 0.2 Micron Diameter Polystyrene Latex Particles.*† (29831)

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Polystyrene latex particles 8000 Å in diameter were utilized in the original Latex Fixation Test(1). Since that time, modifications of the original procedure using latex particles from different sources and of different sizes(2) have resulted in considerable variability of titers from laboratories testing standard rheumatoid arthritis sera. This variation has drawn attention to the need for further standardization of the materials used in this test(3).

The polystyrene latex particles are prepared in various sizes from 1) a latex monomer, 2) a polymerizing catalyst or initiator, 3) water, and 4) an emulsifying agent. Potassium persulfate, potassium percarbonate, potassium perborate, potassium hydroxyperoxide or gamma rays from Co^{60} may serve as the initiator. An anionic emulsifier such as Triton X may be employed in preparation of these particles(4,5). The amount of emulsifier adsorbed on the particles varies with their size and so may affect their serological reactivity.

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The purpose of the investigation reported here was to study some physical properties of latex particles—namely, their stability, both uncoated and coated with human gamma globulin, in the presence of several electrolytes at various ionic strengths and pHs. On the basis of studies reported elsewhere(6,7) latex particles 2000 Å in diameter were used.

Materials. Lytron #615‡ polystyrene latex particles ranging in size between 1500 and 2500 Å in diameter were employed. As obtained from the manufacturer, the particles have the following characteristics: 1) negative charge, 2) a pH of 10.3 ± 2 in an aqueous suspension, 3) a specific gravity of 1.03 and 4) a viscosity of 20 to 40 centipoises at 25°C. They are unchanged by freezing and thawing(8).

The latex suspension was diluted with distilled water until 0.1 ml of the diluted suspension added to 10 ml of water matched the optical density of a standard barium sulfate suspension, as measured in a Coleman Jr. spectrophotometer at 650 mμ. The diluted latex suspension, which contained 30.6 mg of dry latex per ml of solution, was used in the preparation of uncoated latex particles and latex particles coated with gamma globulin. The standard barium sulfate suspension was prepared by adding 3 ml of

‡ Obtained through the courtesy of Monsanto Chemical Co., Springfield, Mass.