THE INCORPORATION OF RADIOACTIVE PHOSPHORUS INTO THE PHOSPHOLIPIDS OF HUMAN LEUKEMIC LEUKOCYTES AND PLATELETS *

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The phospholipid composition of human platelets and erythrocytes has been reported by several investigators (1–4) and the metabolism of phospholipids in human blood has been studied in some detail (5–9). Recent investigations of phospholipid metabolism of leukocytes have been reported (6, 10) but we are not aware of similar studies on platelets.

In this laboratory, investigation of the biochemical characteristics of leukemic leukocytes is being carried out, and as part of this program an examination of the phospholipid metabolism of these cells was undertaken. In this report are presented data on the phospholipid composition of platelets and leukocytes from leukemic patients, and the results of studies on the incorporation of P^{32}-labeled orthophosphate (P^{32}) into the phospholipids of these formed elements *in vivo* and *in vitro*.

The phospholipid composition was similar in leukocytes and platelets from chronic leukemia and in leukocytes from acute leukemia. The pattern of incorporation into leukocyte phospholipids of P^{32} administered therapeutically to patients with chronic myelocytic leukemia was studied, and it was found that rapid incorporation of P^{32} could be demonstrated in lecithin, phosphatidylethanolamine, phosphatidylserine, inositol phosphatide, and sphingomyelin. In the course of this work it was also found that P^{32} is rapidly incorporated into the phospholipids of platelets, but is incorporated only slowly into the phospholipids of erythrocytes.

Observations were made on the *in vitro* incorporation of P^{32} into the phospholipids of platelets and leukemic leukocytes. Active incorporation of P^{32} by all phospholipid components of both formed elements was demonstrated, with the greatest incorporation occurring in inositol phosphatide and a component believed to be phosphatidic acid.

MATERIALS AND METHODS

Patients studied. Two patients with acute myelocytic leukemia, 2 with chronic lymphocytic leukemia, and 9 with chronic myelocytic leukemia were utilized in this study. Seven of the patients had not been treated previously for their leukemia. Six of the patients, all with chronic myelocytic leukemia, had been treated previously by chemotherapy or with P^{32}, but were in relapse with leukocyte counts above 100,000 per mm^3 at the time of the study. There was no apparent difference in the results obtained from previously treated or untreated patients, although the series is too small to permit definite conclusions on this point.

Separation of the formed elements of the blood. Leukocytes were isolated from patients with acute or chronic myelocytic or lymphocytic leukemia with white blood cell counts greater than 100,000 per mm^3. Five hundred ml of blood was collected in a plastic platelet pack (Fenwal) containing 50 ml of 3 per cent ethylenediamine tetraacetic acid (USP) as anticoagulant. All subsequent operations were performed at 4° C. The blood was stored for 1.5 hours to permit sedimentation of the erythrocytes. The plasma layer containing platelets and leukocytes was removed by syphoning into a plastic transfer pack (Fenwal) and then centrifuged in silicone-coated (G.E. Drifilm) 40 ml centrifuge tubes at 180 G for 15 minutes to sediment the leukocytes. The leukocytes were washed 3 times with twice their volume of 0.85 per cent NaCl solution and each time were collected by centrifugation at 180 G for 15 minutes. Platelets were isolated by centrifuging the platelet-rich plasma at 2,500 G for 10 minutes and were washed 3 times with 0.85 per cent NaCl solution. Erythrocytes from the sedimented layer of the

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original plastic bag were collected by centrifugation at 180 G and were washed 3 times with twice their volume of 0.85 per cent NaCl solution. Platelet-poor plasma was obtained by recentrifuging plasma after the isolation of platelets twice at 2,500 G for 15 minutes.

The leukocyte preparations were contaminated with small numbers of erythrocytes and platelets (3 to 4 erythrocytes and 10 to 20 platelets per 100 leukocytes). There was little contamination of the platelet preparation as determined by microscopic study of the suspension stained supravitally with neutral red and Janus green. The erythrocyte preparation usually contained 25,000 to 50,000 platelets per mm^3. Platelet-poor plasma contained 10,000 to 20,000 platelets per mm^3. Platelet counts were done by the method of Brecher and Cronkite (11) using phase contrast microscopy, and erythrocyte and leukocyte counts were done with a standard counting chamber after appropriate dilution.

Preparation of phospholipid extracts. Phospholipid extracts of the above samples were made by adding freshly prepared cell suspensions or plasma slowly to 15 volumes of chloroform: methanol (1:1, vol/vol) at room temperature and then homogenizing the mixture in a Waring blender in an atmosphere of nitrogen for 2 minutes (12). The mixture was allowed to stand at room temperature for 15 minutes and then was filtered. The residue was re-extracted with the same volume of chloroform: methanol (1:1). In preliminary experiments a third extraction yielded 1.1 per cent of the P present in the first two, and for this reason only two extractions were used as a routine. The combined chloroform: methanol extracts were emulsified with an equal volume of water. The mixture was centrifuged and the aqueous layer discarded. In some experiments the water wash was repeated after restoring the organic layer to the original volume with methanol. The organic layer was distilled to dryness under reduced pressure in an atmosphere of nitrogen. The residue was extracted with a small volume of isoamyl alcohol: benzene (1:1, vol/vol) and the lipid solution was stored at − 20° C.

Column chromatography. Phospholipid extracts were chromatographed on columns of silicic acid by the method of Hanahan, Dittmer and Warashina (13). The solvent was removed in a stream of nitrogen from an aliquot of the extract containing 3 to 5 mg of P and the residue was dissolved in a few milliliters of chloroform: methanol (19:1, vol/vol). The solution was applied to a column 1 cm in diameter containing 5 g of silicic acid (Mallinckrodt, AR) which had been heated at 110° C for 18 hours prior to use; 90 to 95 per cent of the applied P was recovered by stepwise elution with chloroform: methanol (19:1, 7:1, 4:1, 3:2, and 1:4, vol/vol). Occasionally the phospholipid was applied to the column in chloroform and the development begun with chloroform followed by the chloroform: methanol mixtures in sequence. The flow rate was usually 10 to 15 ml per hour and 2 to 3 days were required for complete elution of the phospholipids. Fractions of 5 ml were collected and total P was determined on each tube by the method of Fiske and Subbarow (14) using sulfuric acid-hydrogen peroxide to digest the samples. The appropriate tubes were combined and distilled to dryness *in vacuo* in an atmosphere of nitrogen. The residue was dissolved in isoamyl alcohol: benzene, 1:1 and stored at − 20° C.

Analysis of fractions. The components of the fractions from the columns were identified by chromatography at 4° C on silicic acid-impregnated paper using 2,6-dimethyl-4-heptanone: glacial acetic acid: water (40:20:3) as solvent (15). The components were identified by their Rf values and staining reactions with: rhodamine 6G; ninhydrin; 2,4-dinitrophenylhydrazine (15); and the choline spot test (16). The Rf values given in the text are averages derived from many chromatograms. Migration of the components was compared with purified phospholipids obtained from brain and soybean by solvent fractionation and column chromatography (13, 17, 18). Phosphatidic acid was prepared from soybean lecithin purified on an aluminum oxide column (18) using carrot lecithinase and was isolated as the sodium salt (19). A pure sphingomyelin preparation was kindly supplied by Dr. G. V. Marinetti of the University of Rochester.

Further confirmation of the identity of the components of the fractions was obtained by examination of acid hydrolysis products for ethanolamine, serine, choline, and inositol using paper chromatography and the following solvent systems: propanol: water (4:1), butanol: acetic acid: water (4:1:5), and phenol: water (4:1). Hydrolysis products of the phosphoinositide and phosphatidic acid fractions were studied in more detail as outlined below. The fractions were also tested for sterols by the Lieberman-Burchard reaction and for carbohydrate by the Molisch test. No free amino acids were present as indicated by paper chromatography.

Degradation of phosphoinositide and phosphatidic acid. In order to obtain information regarding the chemical nature of the highly-labeled components of the phosphoinositide and phosphatidic acid fractions, these were subjected to mild hydrolysis and the P-containing components identified by paper chromatography and analyzed for radioactivity.

The fractions containing phosphoinositide were hydrolyzed in 2 N HCl at 100° C for 10 minutes (20); the resulting water-soluble P compounds were chromatographed on paper with methanol: formic acid: water (16:3:1), propanol: ammonia: water (5:4:1), and propanol: acetic acid: water (3:1:1) (20, 21). The P-containing compounds were located by the method of Wade and Morgan (22), and were cut from the paper and assayed for radioactivity. Autoradiograms of the paper chromatograms were also prepared.

In experiments to determine the labeled hydrolytic products of the component migrating as phosphatidic acid, carrier soybean phosphatidic acid was added because of the small amount of material in this fraction. Following column chromatography the phosphatidic acid fraction was subjected to mild alkaline hydrolysis at 37° C for 20 minutes (23) and the water-soluble P compounds

were chromatographed on paper and assayed for radioactivity as outlined above.

Quantitative paper chromatography. The quantity of P present in each phospholipid spot was determined by extracting each component from the paper with 1 N methanolic HCl (24) and estimating the total P in the extract by the method of Bartlett (25). Triplicate estimations were made for each component.

Radioactivity measurements. Autoradiograms were prepared from paper chromatograms of the intact phospholipids after staining with rhodamine 6G, and of the P-esters after staining by the method of Wade and Morgan (22). The radioactivity of whole phospholipid extracts was determined by pipetting an aliquot in a thin layer on a metal planchet. The radioactivity of the individual components on the paper chromatograms was determined by cutting out the spots and affixing the paper to a planchet for counting. Correction for self-absorption was made when necessary. A thin window gas-flow counter was used for all determinations of radioactivity. All samples were counted for a sufficient time to give a standard error of less than 2.5 per cent.

In vitro studies. Leukocytes and platelets were prepared as above except that sterile techniques were employed throughout. The cells were suspended in platelet-poor, sterile plasma containing streptomycin, 100 μg per ml, and penicillin, 100 U per ml, and were incubated with $Na_2HP^{32}O_4$ at 37° C, in a stoppered tube in an atmosphere of air, with shaking for an appropriate time. Leukocyte and platelet counts were performed on an aliquot of the suspension taken prior to incubation. At the end of the incubation period the vessels were chilled to 0° C and the cells collected by centrifugation. The sediment was washed 2 or 3 times with ice-cold 0.85 per cent NaCl solution and the phospholipid extract prepared as outlined above.

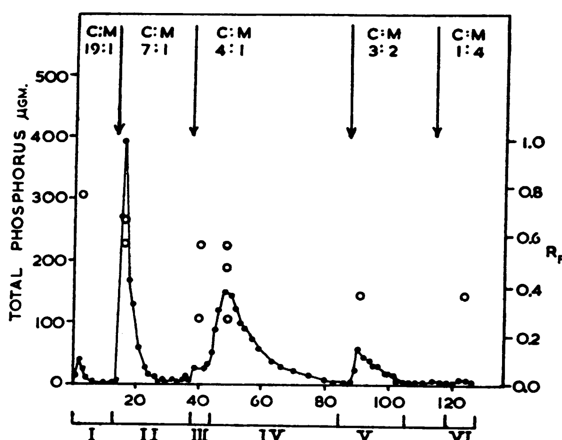

FIG. 1. CHROMATOGRAM OF HUMAN CHRONIC MYELOCYTIC LEUKEMIC LEUKOCYTE PHOSPHOLIPIDS (4.5 MG OF P) ON A 5 G SILICIC ACID COLUMN. The eluting solvents were mixtures of chloroform and methanol and are shown at the top of the figure. The numbers on the abscissa indicate the 5 ml fractions collected and beneath is shown the grouping of the tube contents into the six fractions discussed in the text. The open circles represent the Rf, indicated on the right ordinate, of the components present on paper chromatograms of each peak, as discussed in the text.

RESULTS

Column chromatography. Leukemic leukocytes were obtained from two patients with acute myelocytic leukemia, two with chronic lymphocytic leukemia and nine with chronic myelocytic leukemia. Nineteen column chromatographic analyses were done on leukocyte phospholipids from these patients. Four major peaks were present in the column chromatograms (Figure 1). In some columns a small fifth peak was obtained with the final solvent, chloroform : methanol, 1 : 4.

One to 5 per cent of the total P eluted from the column was present in fraction I which also contained an unidentified yellow pigment and sterols as indicated by a positive Lieberman-Burchard test. On paper chromatography of this fraction two components were present: one migrated just behind the solvent front and probably represented nonphospholipid material, and the other had the Rf (0.77) and staining characteristics of phosphatidic acid.

Fraction II contained 29 to 35 per cent of the P eluted from the column and on paper chromatography contained components behaving as phosphatidylethanolamine (Rf 0.67) and phosphatidylserine (Rf 0.57). The phosphatidylethanolamine contained some plasmalogen as indicated by reaction with 2,4-dinitrophenylhydrazine on the paper chromatograms. The acid hydrolysis products of this fraction contained ethanolamine and serine as demonstrated by paper chromatography. A positive Molisch test for carbohydrate was found with this fraction in some experiments.

Fraction III was the shoulder of the third peak and contained 2 to 4 per cent of the total P eluted. On paper chromatography it contained phosphatidylserine (Rf 0.57) and a phosphoinositide (Rf 0.28). The water-soluble products of acid hydrolysis of this peak contained serine and inositol.

Fraction IV was the remainder of the third peak and contained 53 to 59 per cent of the total P eluted. On paper chromatography of this peak large amounts of phosphatidylcholine (Rf 0.48) and small amounts of phosphoinositide (Rf 0.28) were

detected. On a few occasions a trace of phosphatidylserine (Rf 0.57) was also present. The water-soluble acid hydrolysis products of this peak contained choline and inositol.

Fraction V was usually the final peak eluted. It contained 7 to 10 per cent of the total P. A single component with the Rf (0.37) and staining properties of sphingomyelin was present on paper chromatograms of this peak. Rarely, the fifth fraction also contained a trace of phosphatidylserine and phosphatidylcholine.

Fraction VI when present contained only 0.5 to 1.0 per cent of the total P, and on paper chromatograms contained a single component behaving as sphingomyelin.

Quantitative estimation of the phospholipid components. Aliquots of the fractions obtained from column chromatograms were spotted on silicic acid-impregnated paper and after chromatography the P content of each component was determined in order to calculate the percentage of total P present. Data obtained in this manner from extracts of leukocytes from patients with chronic myelocytic leukemia are shown in Table I. Included also in Table I are data on the phospholipid composition of chronic myelocytic leukemic leukocytes obtained by direct paper chromatography of the whole phospholipid extract. It should be emphasized that with the paper chromatographic method used, only 80 to 90 per cent of the P applied to the paper can be recovered (24); our figures are subject to this limitation.

In vivo labeling of phospholipids by P^{32}. Patients with chronic myelocytic leukemia were given

TABLE I

The phospholipid composition of chronic myelocytic leukemic leukocytes *

	Column fractions	Whole extract
	% total P	
Phosphatidic acid	3	4
Phosphatidylethanolamine	29	29
Phosphatidylserine	6	9
Phosphatidylcholine	46	42
Sphingomyelin	7	10
Phosphoinositide	9	6

* Quantitative paper chromatography was performed on whole phospholipid extracts and on fractions following column chromatography of the extracts. Figures for the column fractions are the average of two columns and for the whole cell extract are the average obtained from five extracts.

TABLE II

Specific activity of whole phospholipid extracts from chronic myelocytic leukemic blood obtained 8 days after the administration of 3 mc of P^{32}

	cpm/µg P
Plasma	21
Leukocyte	30
Platelet	25
Erythrocyte	9

3 mc of $Na_2HP^{32}O_4$ intravenously for therapy, and the incorporation of P^{32} into the blood phospholipids was followed at intervals. No radioactivity was detected in the phospholipids of the formed elements 2 hours after administration of P^{32}, but 18 hours later both leukocyte and platelet phospholipids were labeled, although no radioactivity was detected in the erythrocyte preparation. At 5 days the platelet and leukocyte preparations were strongly labeled and radioactivity had appeared in the red blood cell extracts. The slow appearance of radioactivity in the erythrocyte phospholipids compared with the other components of whole blood is emphasized in Table II wherein are presented the specific activities of the whole phospholipid extracts 8 days after therapy with P^{32}. In Figure 2 are presented autoradiograms of paper chromatograms of the phospholipid extracts of plasma and leukocytes obtained 5 days after administration of P^{32}. The principal components labeled in both plasma and leukocytes were phosphatidylethanolamine, phosphatidylcholine, and phosphoinositide, and faint labeling could be detected in sphingomyelin. In addition, leukocyte phosphatidylserine was faintly labeled. The labeling pattern was confirmed on phospholipid components separated by column chromatography prior to paper chromatography. A similar labeling pattern was found for platelet phospholipids from the patients.

In Table III are presented the specific activities of the phospholipid components from leukocytes obtained 6 days after administration of P^{32}. These data confirm the impressions obtained from the autoradiograms. No radioactivity was present in the component believed to be phosphatidic acid, and phosphatidylserine and sphingomyelin had approximately half the specific activity of the other components.

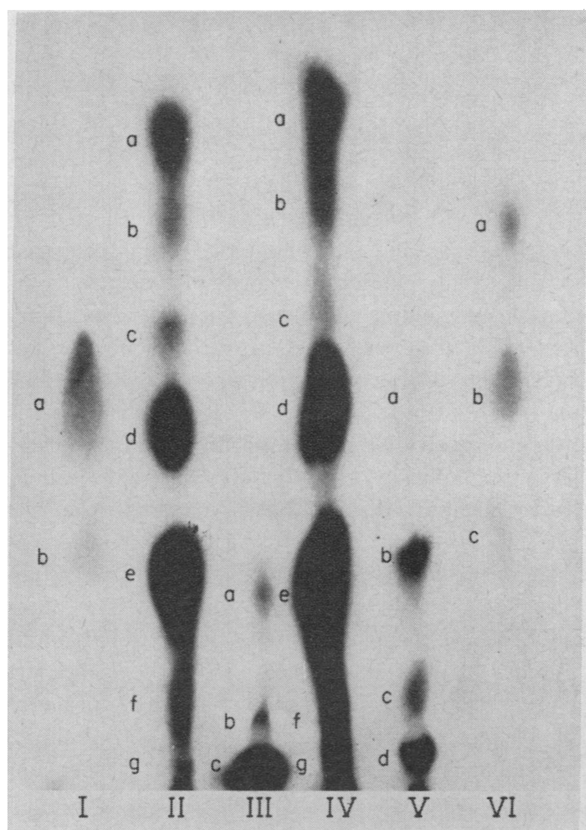

FIG. 2. AUTORADIOGRAMS OF PAPER CHROMATOGRAMS OF WHOLE PHOSPHOLIPID EXTRACTS OF BLOOD COMPONENTS EXPOSED TO P^{32} *IN VIVO* AND *IN VITRO*. Six µg of P was applied for each preparation; the film was exposed for 4 weeks. Lanes I and VI are from *in vivo* experiments and lanes II to V are from *in vitro* experiments. The bottom of the figure represents the starting line and the top the solvent front.

I. Extract of plasma from a patient with chronic myelocytic leukemia 5 days after P^{32} therapy (3 mc); a, phosphatidylcholine; b, sphingomyelin and phosphoinositide. These last two components were separated when stained with rhodamine 6G and examined under ultraviolet light, but on the autoradiograms they appear as a combined spot. Both were labeled when examined after separation by column chromatography. In addition, a faint spot with Rf of phosphatidylethanolamine could be seen on the original autoradiogram.

II. Extract of chronic myelocytic leukemic leukocytes after 6 hours' incubation of 7.5 ml of a suspension of 188,000 leukocytes and 36,000 platelets per mm³ with 0.5 mc of $Na_2HP^{32}O_4$; a, phosphatidic acid; b, phosphatidylethanolamine; c, phosphatidylserine; d, phosphatidylcholine; e, phosphoinositide; f, unidentified component; g, inorganic P.

III. Plasma extract from II; a, phosphoinositide; b, unidentified component; c, inorganic P.

IV. Extract of platelets from a patient with chronic myelocytic leukemia after 6 hours' incubation of 7.5 ml of a suspension of 2,860,000 platelets per mm³ with 0.5 mc of $Na_2HP^{32}O_4$. Identity of the components as in II. There was marked trailing of the highly-labeled phosphoinositide but on the original autoradiogram, spots f and g were clearly visible.

V. Plasma extract from IV; a, phosphatidylcholine; b, phosphoinositide; c, unidentified component; d, inorganic P.

VI. Extract of leukocytes from a patient with chronic myelocytic leukemia 5 days after P^{32} therapy (3 mc); a, phosphatidylethanolamine; b, phosphatidylcholine; c, phosphoinositide and sphingomyelin. In addition, a faint phosphatidylserine spot could be detected on the original autoradiogram.

TABLE III

Comparison of the incorporation of P^{32} into leukocyte phospholipids exposed to $Na_2HP^{32}O_4$ in vivo and in vitro *

	In vivo	*In vitro*
	cpm/µg P	*cpm/µg P*
Phosphatidic acid	0	3,300
Phosphatidylethanolamine	68	230
Phosphatidylserine	32	320
Phosphatidylcholine	70	450
Sphingomyelin	29	500
Phosphoinositide	83	10,600

* The data from the *in vivo* experiment were obtained from an extract of chronic myelocytic leukemic leukocytes obtained 6 days after the intravenous administration of 3 mc of $Na_2HP^{32}O_4$ to a patient. Paper chromatograms of the whole extract were used for determination of radioactivity and P. The reaction mixture for the *in vitro* experiment contained 0.5 mc of $Na_2HP^{32}O_4$, penicillin 750 U, and streptomycin 750 µg in 7.5 ml of a suspension of 188,000 leukocytes and 36,000 platelets per mm³ from a patient with chronic myelocytic leukemia. The vessel was incubated for 6 hours at 37° C. The data presented were obtained by paper chromatography of the whole phospholipid extract.

In vitro studies of exchange of leukocyte and plasma phospholipids. It has been suggested that some plasma phospholipids are synthesized in the cells of the peripheral blood, and that plasma phospholipids are readily incorporated into the phospholipids of blood cells (7, 8, 10). In order to obtain evidence bearing on this possibility, experiments were performed using leukemic leukocytes and plasma containing phospholipids labeled with P^{32} *in vivo*. In one experiment a suspension of leukemic leukocytes labeled with P^{32} was sedimented at 2,500 G for 15 minutes and the packed cells were resuspended in 5 ml of unlabeled platelet-poor plasma. The platelet count of the final suspension was 90,000 per mm³ and the packed cell volume was 11 per cent as determined in duplicate

by centrifuging an aliquot of the suspension in a capillary tube at 12,000 G for 10 minutes. The suspension was incubated at 37° C and at 0 and 6 hours 2-ml aliquots were chilled in ice and the cells and plasma separated by centrifugation at 2,500 G for 15 minutes. The plasma was recentrifuged at 2,500 G for 15 minutes, and the leukocytes were washed 3 times with 0.85 per cent NaCl solution. Phospholipid extracts were prepared from plasma and cells and the radioactivity present in each was determined. At 0 time plasma phospholipids contained 5 per cent of the radioactivity of the leukocyte phospholipids and at 6 hours contained 15 to 18 per cent of the radioactivity of the leukocyte phospholipids. In a converse study done in the same manner, a suspension of previously unlabeled leukemic leukocytes was incubated in 5 ml of labeled plasma. The packed cell volume of the suspension in this experiment was also 11 per cent. At both 0 and 6 hours the phospholipids of the cells contained 3 per cent of the radioactivity present in the phospholipids of the plasma.

Studies on incorporation of P^{32} into leukocyte and platelet phospholipids in vitro. When suspensions of leukemic leukocytes or platelets from normal or leukemic patients were incubated with $Na_2HP^{32}O_4$ and the phospholipids extracted and analyzed for radioactivity, it was found that there was rapid incorporation of radioactivity in all of the phospholipid components. The experimental details are given with Figure 2 and Table III. In a typical experiment the whole phospholipid extract contained 0.14 per cent of the total radioactivity added to the reaction mixture. No incorporation of P^{32} was found in the phospholipids of leukocytes or platelets of reaction mixtures sampled at 0 time. The incorporation of P^{32} into both platelet and leukocyte phospholipids increased with time, and reached a plateau at 4 to 6 hours. Incubation for 6 hours with P^{32} of a suspension in plasma of platelets damaged by freezing and thawing resulted in no incorporation of radioactivity into the phospholipids.

Since all of the leukocyte preparations were contaminated with some platelets, the possibility that the P^{32} incorporation might be entirely due to their presence was considered. Leukocytes and platelets from a patient with chronic myelocytic leukemia were employed. The incorporation of P^{32} into the phospholipids was compared in a suspension containing 522,000 leukocytes per mm^3 and 53,000 platelets per mm^3 with that in a suspension containing 210,000 platelets per mm^3 and very few leukocytes. Five-ml aliquots of these preparations were incubated in duplicate for 6 hours with 0.4 mc of $Na_2HP^{32}O_4$. At the end of this time the formed elements were collected by centrifugation and phospholipid extracts prepared in the usual manner. In order to ensure comparable extraction conditions for all vessels, carrier leukocytes were added after the incubation period to the vessels containing platelets alone. The phospholipid extract from the leukocyte preparation contained 0.87 mg P and that from the platelets after addition of carrier contained 0.94 mg P. Twice as much radioactivity was present in the phospholipid extract from the leukocytes as was present in the extract from the platelets. Assuming that the incorporation of P^{32} into the phospholipids of the platelets was proportional to the number of platelets in each vessel, the leukocytes were responsible for approximately 87 per cent of the incorporation of P^{32} into the phospholipids extracted from the leukocyte-platelet suspension.

The incorporation of P^{32} into the phospholipids of platelets and leukocytes from patients with leukemia is illustrated in Figure 2 by autoradiograms of paper chromatograms of phospholipid extracts. The pattern of incorporation of P^{32} into the phospholipids of normal platelets was similar to that found with platelets from patients with leukemia but no detailed quantitative comparison was made. No studies were done with normal leukocytes. The autoradiograms show that the phospholipids of the platelets were more intensely labeled than those of the leukocytes. All of the radioactive spots on the paper chromatograms could be identified except that labeled "f" in Figure 2. This component migrated with Rf 0.11 and was not detected with rhodamine 6G, ninhydrin, or the choline stain. Its identity is unknown but from its Rf it is possibly a nonlipid phosphorus compound (26, 27). Lysolecithin (4) and a phosphoinositide (28) have been reported to migrate with approximately this Rf but would have been detected with rhodamine 6G. Included in Figure 2 are autoradiograms of extracts of the plasma from the leukocyte and platelet suspensions incubated with P^{32}. The radioactivity was principally in the phosphoinositide component. The specific

activities of the plasma phospholipids in these *in vitro* experiments were less than 2 per cent of the specific activities of the leukocyte or platelet phospholipids.

In Table III are presented the specific activities of the components of the phospholipid extract from the leukemic leukocytes. The specific activities of the components migrating as phosphatidic acid and phosphoinositide were 6 to 40 times higher than those of the other components.

Because of their high specific activities the phosphoinositide and phosphatidic acid fractions were studied further in order to determine the chemical nature of the radioactive P present. The phosphoinositide fraction was subjected to acid hydrolysis and the water-soluble phosphate compounds released were separated by paper chromatography. The principal labeled compound migrated identically with authentic inositol monophosphate (California Corp. for Biochemical Research, Inc.). Some glycerophosphate and inorganic P were also present. The specific activity of the intact phosphoinositide was 390 cpm per μg P and that of the inositol monophosphate isolated from the hydrolysate was 410 cpm per μg P. These results indicate that the P^{32} in this component was in chemical combination with inositol and that the parent compound was a monophosphoinositide, probably phosphatidylinositol.

Attempts to identify the labeled component migrating as phosphatidic acid were carried out using the technique of co-chromatography (29). Relatively large amounts of unlabeled soybean phosphatidic acid were mixed with the labeled phosphatidic acid fraction and chromatographed on silicic acid-impregnated paper. The dried chromatogram was stained with rhodamine 6G to detect the phospholipid spots and an autoradiogram was prepared. The radioactivity was present in the phosphatidic acid spot and extended nearly, but not entirely, to the margin of the area stained with rhodamine 6G. As postulated by Hokin and Hokin (29) this result suggests that the unknown compound is similar to, but not identical with, the phosphatidic acid derived from soybeans. The difference may be due to differences in the fatty acids of the two compounds.

The phosphatidic acid fraction was also subjected to mild alkaline hydrolysis and the water-soluble phosphate compounds were separated by paper chromatography. The only labeled phosphate compound detected by direct counting or radioautography was glycerophosphate, suggesting that it was derived from labeled phosphatidic acid (23, 29, 30), rather than polyglycerophosphatide (31–34).

DISCUSSION

No significant qualitative difference was noted in the phospholipid composition of leukocytes obtained from patients with acute leukemia or chronic lymphocytic or myelocytic leukemia. The phospholipid composition of platelets was similar to that found for leukemic leukocytes. The analytical data agree essentially with those of others for platelets and erythrocytes (4). The presence of the component believed to be phosphatidic acid which constituted up to 4 per cent of the total phospholipids of both platelets and leukocytes is of particular interest because of its rapid incorporation of P^{32} *in vitro*. This component has been referred to as phosphatidic acid because of its behavior on co-chromatography with soybean phosphatidic acid and the finding that only glycerophosphate is formed on mild alkaline hydrolysis of the component. Troup, Reed, Marinetti and Swisher (4) have reported the presence of a phospholipid component of extracts of platelets and erythrocytes which they identified as a mixture of polyglycerophosphatides, including phosphatidic acids. It is possible that some polyglycerophosphatides were present in the small quantity of material studied here but were not detected. Both phosphatidic acids and polyglycerophosphatides have been identified in animal tissues (23, 29, 31–35).

The ability of the formed elements of blood to synthesize lipids has been studied by James, Lovelock and Webb (7) and the biosynthesis of phospholipids by whole blood has been studied by Rowe (9). Others have concluded that the leukocyte is the major site for lipid synthesis in blood (10) and that mature erythrocytes are not capable of synthesizing lipids (6, 10). The studies reported here indicate that platelets and leukemic leukocytes synthesize phospholipids also. Pure platelet preparations containing 200,000 platelets per mm^3 incorporated half as much P^{32} into phospholipid as a leukemic leukocyte preparation containing 500,000 leukocytes and 50,000 platelets per mm^3. It

could be inferred that platelets are the major site of incorporation of P^{32} into phospholipids *in vitro* in whole blood containing 10,000 leukocytes and 250,000 platelets per mm^3.

It has been suggested that plasma phospholipids may be synthesized in cells of the peripheral blood and that phospholipids of plasma may be readily incorporated into the phospholipids of blood cells (7, 8, 10). In experiments in which unlabeled leukemic leukocytes were incubated with labeled plasma no significant transfer of labeled phospholipid from plasma to cells was observed, whereas in the converse experiment a small amount of phospholipid was transferred from previously labeled leukemic leukocytes to plasma. The phospholipid appearing in the plasma may have been actively transported from living cells, or may have been released from leukocytes or contaminating platelets which lysed during the incubation period. In the experiments in which leukocytes or platelets suspended in plasma were incubated with P^{32} *in vitro* the small amount of radioactivity present in the phospholipids extracted from plasma could most probably be accounted for by platelets remaining in the plasma after centrifugation. Thus no definite evidence was obtained which might relate the metabolism of phospholipids in leukemic leukocytes and plasma.

In the *in vitro* experiments P^{32} was incorporated into all the phospholipid components of the leukocytes and platelets. The highest specific activities were attained by the component believed to be phosphatidic acid and by a phosphoinositide which probably has the structure of phosphatidylinositol. These findings are similar to those of Dawson (36) using guinea pig brain and those of Hokin and Hokin (37, 38) using pigeon pancreas and guinea pig brain. Rowe (9) reported that cephalin was the most highly labeled phospholipid component in whole blood incubated with P^{32}. This author did not identify phosphatidic acid or phosphoinositide as components of the phospholipid extracts, and it is possible that these compounds were responsible for much of the radioactivity present.

There is a striking difference between the labeling pattern of the phospholipids of leukocytes and platelets from patients receiving P^{32} therapeutically and the labeling when the cells are incubated with P^{32} *in vitro*. Label could be detected in the characteristic *in vivo* pattern in the leukocytes and platelets of patients 18 hours after injection of P^{32}. The *in vitro* incubations were usually performed for only 6 hours but when the incubation was extended to 18 hours the difference in pattern persisted, indicating that duration of exposure of the cells to P^{32} does not account for this result. The difference may reflect the persistent high level of P^{32} *in vitro* which could permit detection of components present in small quantity but having a rapid turnover of P. Another possibility is that P^{32} is incorporated into younger cells *in vivo* and the pattern of phospholipid synthesis may be directed more toward structural phospholipids than toward those with other physiological functions in young cells.

Dawson (39) has reported that the pattern of incorporation of P^{32} into phospholipids was different in guinea pig liver and brain when the P^{32} was administered *in vivo* and when slices of these organs were incubated with P^{32} *in vitro,* and Marinetti and co-workers (26, 27) have reported differences in the labeling pattern of phospholipids from rat liver when the P^{32} was administered *in vivo* and when homogenates of rat liver were incubated with P^{32}.

The incorporation of P^{32} from $Na_2HP^{32}O_4$ into the phosphoinositide and phosphatidic acid components of leukocytes and platelets incubated *in vitro* probably represents, at least in part, exchange of the phosphate moiety of the molecule rather than true synthesis of new phospholipid. From studies on the effect of acetylcholine on the turnover of P^{32} in phospholipids of various organs, Hokin and Hokin (40–42) have postulated that the turnover is related to transport of material across the cell membrane. Perhaps the turnover of P^{32} in some phospholipid components of leukocytes and platelets is related to the same phenomenon. It is also possible that this finding is related to the postulated role of phosphatidic acid as an intermediate in phospholipid synthesis (43). In any case the results obtained with platelets are evidence that these formed elements have very active phospholipid metabolism which must be included in any consideration of platelet physiology or disease.

SUMMARY

1. The phospholipid composition of platelets and leukocytes from patients with acute and chronic leu-

kemia has been studied by a combination of column and paper chromatography. The phospholipid composition of these formed elements was similar and included phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, sphingomyelin, a phosphoinositide, probably phosphatidylinositol, and a component believed to be phosphatidic acid.

2. The incorporation of P^{32} into the leukocyte and platelet phospholipids of patients given $Na_2HP^{32}O_4$ therapeutically has been studied. Radioactivity was present in the phospholipids 18 hours after administration of P^{32} and was found principally in phosphatidylethanolamine, phosphatidylcholine, and the phosphoinositide.

3. The *in vitro* incorporation of P^{32} into the phospholipids of leukemic leukocytes and platelets was also studied. The major incorporation of radioactivity occurred in the phosphoinositide and a component believed to be phosphatidic acid, in contrast to the pattern of labeling found *in vivo*.

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