2. The ribonuclease and also proteolytic enzymes were removed from yeast extracts by adsorption on bentonite.

3. The consecutive use of Zn^{2+} ions and bentonite provided a mild method for the isolation of ribonucleic acids and ribonucleoprotein in which the ribonucleic acids had not been enzymically degraded.

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The Biosynthesis of Phospholipids by Human Blood Cells

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For studies on the phospholipids of human blood cells information is required both on the chemical structures of the individual molecules and on their respective metabolic activities. Although the functions of cellular phospholipids are not well defined, these compounds form an integral part of all cell membranes. Finean (1957) has discussed the structure of nerve myelin, in which the ratio of cholesterol:phospholipid:cerebroside is 2:2:1. Lovelock (1955*a*) showed that phospholipid diffuses readily from human blood cells in considerable quantities when these are washed or treated with alumina. Moreover, a constant phospholipid:cholesterol ratio is necessary for the maintenance of the integrity of the cells (Lovelock, 1955*b*). The differences, if any, between the roles of the different classes of phospholipids, e.g. kephalins, lecithins and sphingomyelins, are still obscure.

It has been shown that ^{14}C-labelled acetate is incorporated into rabbit blood cells *in vivo* (Altman, Whatman & Salomon, 1951) and *in vitro* (Altman, 1953). James, Lovelock & Webb (1957) showed that acetate was incorporated into the fatty acids of human blood cells *in vitro*. The incorporation of [^{32}P]orthophosphate *in vivo* into phospholipids of human blood cells has been studied (Tuttle, Scott & Lawrence, 1939; Erf, Tuttle & Lawrence, 1941; Lawrence, Erf & Tuttle, 1941), but there appears to have been little work on the incorporation *in vitro*.

In the following experiments two approaches to lipid metabolism were combined and the concomitant incorporations of [^{32}P]orthophosphate and [Me-^{14}C]acetate into the phospholipids of human blood cells were studied. The separation of human blood cellular phospholipids into the main classes by chromatography on silicic acid is described. Analyses were made of the fatty acids in each class. Moreover, an attempt was made to measure the metabolic activity of each class both from the point of view of the fatty acid chains and the phosphate groups.

METHODS

Incubation of blood. Freshly drawn whole blood was added to a mixture of acid–citrate–dextrose (0·73 M-trisodium citrate, 0·35 M-citric acid, 0·1 M-glucose; 15 ml./100 ml. of whole blood); [^{32}P]orthophosphate (20–50 μc/ml.); sodium [Me-^{14}C]acetate (3–10 μc/ml., specific activity

13·7 μc/mg.); ethylenediaminetetra-acetic acid (1 drop of 0·1% soln./100 ml. of blood); penicillin (100 i.u./ml.) and streptomycin (100 i.u./ml.). Sterile procedures were used throughout. The mixture was incubated at 34·5° with gentle agitation.

Relative rates of phosphate and acetate incorporations. Whole blood was incubated with radioactive phosphate and acetate as described above. The total volume was 140 ml. Samples (10 ml.) were withdrawn at different time intervals (Fig. 1). The cells were separated by centrifuging, washed with 0·9% sodium chloride soln. (3 ml.) and poured slowly into 100 ml. of ethanol–ether (2:1, v/v). After 24 hr. at room temperature the mixture was stored at 2° until required. Solid matter was removed by filtration and the extract concentrated to dryness *in vacuo* in a rotary evaporator. The residue, which was obtained as a thin film on the inside of the flask, was washed with acetone (1 ml.) which was removed after 5 min. This was repeated once. The acetone extracts, which contained only about 2% of the lipid phosphorus, were not investigated further. The residue was washed with ethanol (2 ml.), which was removed after 5 min. This was repeated once and the flask again washed with ethanol (1 ml.). The white insoluble residue suspended in the ethanolic extract was removed by centrifuging. A portion of the ethanolic extract was evaporated to dryness on a weighed aluminium planchet which was then reweighed. The radioactivity of the sample was then estimated.

The counts from ^{14}C indicated in Fig. 1, where the phospholipid was estimated by weighing, were corrected for self-absorption.

Preparation of phospholipids for chromatographic analysis. Whole blood was incubated with radioactive isotopes as described above. The total volume was approx. 100 ml. After 6·5 hr. the cells were separated by centrifuging, washed with 0·9% sodium chloride soln. (20 ml.) and poured slowly into 1 l. of ethanol–ether (2:1, v/v). After 24 hr. at room temperature the solid matter was removed by filtration and concentrated to dryness *in vacuo* in the rotary evaporator. The residue was washed with acetone (10 ml.), which was removed after 5 min. This was repeated once. Ethanol (10 ml.) was added and removed after 5 min. The residue was extracted again in this manner with 10, 20 and 10 ml. of ethanol respectively. The combined ethanolic extracts were centrifuged to remove white suspended residue and then accurately diluted to 50 ml. This extract contained 0·19 m-mole of phosphorus.

Chromatography on silica-impregnated paper. Phospholipids containing 0·19 μmole of phosphorus were chromatographed on silica-impregnated paper with 20% (v/v) of methanol in chloroform as described by Lea, Rhodes & Stoll (1955). The separated components were detected by exposing the paper to iodine vapour. Nitrogenous kephalins were detected by spraying with ninhydrin followed by heating at 95° for 15 min. Choline-containing lipids were detected by development with phosphomolybdic acid followed by reduction with stannous chloride (Chargaff, Levine & Green, 1948).

Radioautography. Kodirex X-ray films were used and the chromatogram was kept in contact with the film for approximately 40 days.

Chromatography on silica-gel column. The method was that described by Hanahan, Dittmer & Warashina (1957), silica gel prepared as described by Lea *et al.* (1955) being used. The column (25 in. × 1 in. diam.) contained 150 g. of silica gel. Phospholipid (0·17 m-mole of phosphorus; approx. 140 mg.), extracted from the cells from approx. 100 ml. of whole blood, was fractionated. Eluate fractions (approx. 8 ml.) from the column were diluted accurately to 10 ml. and 0·1 ml. was transferred to a polythene planchet and evaporated to dryness. The radioactivity of each fraction was estimated (Fig. 2).

Estimation of radioactivity. Samples of combined fractions (Fig. 2) were transferred to polythene planchets, evaporated to dryness and counted with an end-window Geiger tube. The samples were then recounted with the planchets shielded from the Geiger tube by a calibrated foil of phosphorbronze. Previous calibration established that the shield prevented the ^{14}C β particles from being counted and reduced the counts from ^{32}P by a factor of 0·894. From the two measurements and this factor the counts due to ^{14}C and ^{32}P respectively were calculated. The sample was quantitatively washed off the planchet and the phosphorus estimated by the method of Fiske & Subbarow (1925). A calibration curve was constructed with blood phospholipid containing ^{14}C. This indicated that, assuming an average molecular weight of 800, the self-absorption of β particles from ^{14}C was negligible.

Methanolysis of phospholipids. Dry phospholipid dissolved in anhydrous methanolic 7% (w/v) hydrogen chloride was heated at 104° in a sealed tube for 3 hr. After neutralization with excess of Ag_2CO_3 the mixture was filtered. The solid was washed twice with 1 ml. portions of methanol and then twice with 1 ml. portions of water. After addition of water (8 ml.) the combined filtrate and washings were extracted four times with 4 ml. portions of light petroleum (b.p. 40–60°). More light petroleum (4 ml.) was added to the extracts, which were then dried over anhydrous sodium sulphate.

Detection of the bases. The aqueous extract after methanolysis and extraction with petroleum was concentrated to dryness and the residue extracted twice with 0·5 ml. of methanol. The methanolic extract was concentrated to 0·2 ml. and chromatographed on Whatman no. 1 paper with the solvents butanol–acetic acid–water (63:10:27, by vol.) and phenol–water (5:2, v/v) in an atmosphere of ammonia. Bases were detected by treating the chromatogram with ninhydrin or with phosphomolybdic acid and stannous chloride (Chargaff *et al.* 1948).

Analysis of the fatty acid and methyl esters. These were separated and estimated by gas chromatography with a column of Celite coated with ethylene glycol–adipic acid resin (Lipsky & Landowne, 1958; James, 1958). Argon was the carrier gas and the column was fitted with an ionization detector (Lovelock, 1958) and automatic recorder.

RESULTS

Over the course of 10 hr. both phosphate and acetate were incorporated into the phospholipid fraction (Fig. 1). Chromatography of the doubly labelled phospholipids on silica-impregnated paper showed the presence of three components with R_F values and staining properties corresponding to kephalin (R_F 0·92), lecithin (R_F 0·75) and sphingomyelin (R_F 0·55). Radioautography revealed that most of the radioactivity was in the region of the

kephalin. Lecithin was strongly radioactive and there was a very small quantity of radioactivity in a position corresponding to sphingomyelin. A strongly radioactive component remained at the origin. That the radioactivity detected was due to ^{32}P (half-life 14·3 days) and not to ^{14}C (half-life 5030 years) was demonstrated by repeating the radioautography 7 weeks later, when it was found to have disappeared. The radioactivity due to ^{14}C was not detectable under the conditions used.

A larger quantity (approx. 140 mg.) of the doubly labelled phospholipids, obtained after incubation of blood with the radioactive isotopes for 6·5 hr., was fractionated on a column of silica gel, the fractions being eluted with chloroform–methanol mixtures. The radioactivities of the eluate fractions are shown in Fig. 2. The fractions were grouped according to their radioactivities into 12 major fractions as shown. Fraction 12 was obtained by extracting the extruded column at room temperature with methanol. The yields and the relative incorporations of phosphate and acetate into these fractions are given in Table 1.

The fractions were subjected to methanolysis and the bases in each identified by chromatography on paper. Ethanolamine, after treatment with methanolic hydrogen chloride, yielded one component detectable after chromatography and treatment with ninhydrin [R_F 0·20 in butanol–acetic–acid water (solvent *A*) and R_F 0·91–0·92 in phenol–ammonia (solvent *B*)]. This was indistinguishable from ethanolamine and was detected in the products of methanolysis of fractions 3, 4 and 5. Similar treatment of serine yielded two components (R_F 0·10 and 0·20 in *A*, 0·40 and 0·91 in *B*). The first of these, which was the major product, was

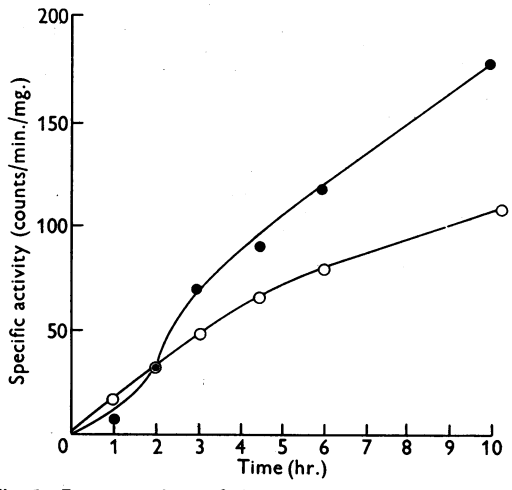

Fig. 1. Incorporation of inorganic [^{32}P]phosphate and [Me-^{14}C]acetate into the phospholipids of human blood cells. ●, Counts due to ^{32}P; ○, counts due to ^{14}C.

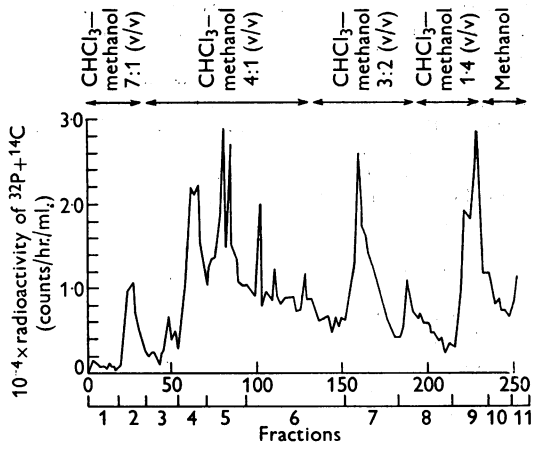

Fig. 2. Chromatography on a column of silica gel of phospholipids from human blood cells. The numbers on the upper abscissa indicate the number of fractions (volume 8 ml. approx.) collected. The numbers on the lower abscissa indicate how these fractions were grouped.

Table 1. *Incorporation of phosphate and acetate into phospholipid fractions of human blood cells*

Fraction	Yield of phosphate (μmoles)	Base	Specific activities (counts/min./μmole of phosphate) ^{32}P	Specific activities (counts/min./μmole of phosphate) ^{14}C	10^{-3} × Total counts (yield × specific activity) ^{32}P	10^{-3} × Total counts (yield × specific activity) ^{14}C	Total counts from ^{32}P / Total counts from ^{14}C
1	1·0	—	0	0	0	0	—
2	1·0	—	5100	466	5·10	0·47	11·0
3	17·4	Ethanolamine	442	81	7·68	1·41	5·5
4	12·5	Ethanolamine	3620	300	45·2	3·75	12·1
5	3·5	Ethanolamine and serine	15700	1220	54·9	4·27	12·9
6	10·0	Serine	8880	800	88·8	8·00	11·1
7	35·0	Choline	980	100	34·2	3·50	9·8
8	20·5	Choline	930	160	19·1	3·28	5·8
9	7·5	Choline, serine and others	2770	330	20·8	2·48	8·4
10	2·0	Choline, serine and others	500	480	1·0	0·96	10·4
11	1·0	Choline and others	560	350	0·56	0·35	1·6
12	4·0	Choline, serine and others	4410	100	17·6	0·40	44·0

Table 2. *Analyses of fatty acids of phospholipid fractions from human blood cells*

Acid	Fractions (see Table 1 and Fig. 2) 3	4	5	6	7	8	9	12
Lauric	—	—	—	1·8	—	—	0·7	1·9
Myristic	—	—	—	—	2·3	1·5	1·4	5·9
Palmitic	2·7	22·6	19·8	11·4	5·2	30·2	29·2	50·9
Unknown	—	—	—	5·4	—	—	—	—
Stearic	18·2	14·7	19·6	17·7	2·2	9·6	8·3	16·2
Oleic	29·2	30·1	22·9	—	5·0	7·9	4·2	15·9
Linoleic	10·5	15·0	6·2	3·1	53·4	7·6	30·6	6·3
Arachidic	—	—	—	—	—	3·1	2·1	—
C_{20} unsaturated (i)	—	—	—	—	—	—	1·6	—
C_{20} unsaturated (ii)	—	—	13·9	—	—	—	—	—
Arachidonic	39·5	17·8	8·3	5·7	22·7	—	—	—
Behenic	—	—	9·4	55·1	—	7·6	4·6	2·2
C_{22} unsaturated	—	—	—	—	—	—	—	—
C_{24} unsaturated (i)	—	—	—	—	—	16·4	5·5	—
C_{24} unsaturated (ii)	—	—	—	—	—	16·2	8·3	—
C_{24} unsaturated (iii)	—	—	—	—	4·9	—	—	—
C_{24} unsaturated (iv)	—	—	—	—	4·3	—	—	—

indistinguishable from serine. The second was indistinguishable from the methyl ester of serine. Serine was detected in the methanolysates of fractions 5, 6, 9, 10 and 12. An unknown ninhydrin-reacting component of R_F 0·13–0·14 (*A*) and 0·57–0·60 (*B*) was detected in fractions 9, 10 and 12. In addition to this, fraction 9 contained two components of R_F 0·33 and 0·47 (*A*) and 0·71 and 0·82 (*B*). The latter component contained choline. Fraction 12 contained a component of R_F 0·17 (*A*) and 0·63 (*B*), which gave a yellow stain when treated with ninhydrin.

The products of methanolysis of fractions 7, 8, 10 and 12 contained choline and chromatography of these indicated the presence of a choline-containing component (R_F 0·24–0·26) distinguishable from choline in *A* (R_F 0·15–0·17) but not in *B* (R_F 0·89–0·90). A choline-containing component which did not contain phosphate but had the same R_F values was obtained from the methanolysis of egg lecithin and the treatment of choline with methanolic hydrogen chloride at 110° for 17 hr. It seems likely, therefore, that this component was methoxycholine. Fraction 11 contained choline and components which gave a purple stain with ninhydrin, but there was insufficient material for chromatographic analysis. Fractions 7 and 8 were distinguished by chromatography on silica-impregnated paper and found to contain choline and sphingomyelin respectively. The distribution of the fatty acids up to the C_{24} acids in each fraction are shown in Table 2.

DISCUSSION

The chromatography on a silica-gel column gave separations into ethanolamine-containing kephalins, serine-containing kephalins, phosphatidylcholine and sphingomyelin. The first two classes came off the column in the reverse order to that found by Hanahan *et al.* (1957) for liver phospholipids. The ratios of the yields, expressed as moles of phosphate, of kephalin, lecithin and sphingomyelin (44:35:21) were close to the molar yields obtained by Hack (1947) from phosphate and choline estimations on unfractionated blood phospholipids.

There were large differences in the distribution of the fatty acids. The most abnormal distribution was shown by the serine-containing lipids, which contained very little unsaturated fatty acids. These serine-containing lipids (fraction 6) had a very high metabolic activity and also a high proportion (55·1%) of an acid with a retention volume corresponding to behenic acid. Fractions 3 and 4, which both contained ethanolamine, were different; fraction 4 had over eight times as much palmitic acid and half as much arachidonic acid as fraction 3.

All the fractions isolated contained both ^{32}P and ^{14}C and therefore contained lipids which had been metabolically active, the activity involving both the phosphate- and carbon-containing parts of the molecules. When phosphate incorporation into each fraction is considered, the kephalins contained 67% of the isolated phospholipid ^{32}P; the lecithins contained 12%, the sphingomyelins 6·5% and fraction 9, 7%. The acetate incorporations were in the same order, 54% being in the kephalins, 11% in the lecithins, 10% in the sphingomyelins and 7·6% in fraction 9. Fraction 9 could be an unknown phospholipid as it contained choline, serine and other substances which stained purple with ninhydrin. It did not haemolyse red cells, and was therefore unlikely to contain appreciable lysophosphatides.

Two different ethanolamine-containing fractions were isolated (fractions 3 and 4) (Table 1). Fraction 4

had 5·9 times the phosphate and 2·7 times the acetate incorporation of fraction 3. Both these fractions developed strong purple colours when treated with Feulgen reagent, indicating the presence of plasmalogens (Feulgen & Bersin, 1939). Collins & Wheeldon (1957) isolated two classes of mixtures of methyl esters of dinitrophenylkephalins from both rat liver and egg yolk. The first class had a phosphorus:dinitrophenol ratio of 1·0 and the second of 2·0. The second class, when derived from rat-liver homogenates incubated with [^{32}P]phosphate, had incorporated more radioactivity than the first class or the corresponding choline-containing lipids. It is possible that the same complexities of kephalin structure occur in human blood cells. In these studies no attempts were made to distinguish between the cellular components of blood, e.g. red cells, white cells and platelets, and it is possible that the different contributions of these may be partly responsible for the metabolic heterogeneity observed.

SUMMARY

1. The simultaneous incorporation *in vitro* of [^{32}P]orthophosphate and [Me-^{14}C]acetate into the phospholipids of human blood cells has been measured.

2. The doubly labelled phospholipids obtained after incubation with the radioactive isotopes for 6·5 hr. were fractionated by chromatography on silica gel, into ethanolamine-containing kephalins, serine-containing kephalins, phosphatidylcholine, sphingomyelin and other phospholipids.

3. All fractions were labelled with both ^{32}P and ^{14}C. Of the isolated phospholipid ^{32}P the kephalins contained the most (67 %), followed by the lecithins (12 %) and the sphingomyelins (6·5 %). The incorporation of ^{14}C followed the same order, the kephalins containing 54 %, the lecithins 11 % and the sphingomyelins 10 % of the isolated lipid ^{14}C.

4. The fatty acids of the fractionated phospholipids were analysed by vapour-phase chromatography and different distributions of fatty acids for each fraction were observed.

5. Two ethanolamine-containing phospholipid fractions were isolated. These differed in the rates of phosphate and acetate incorporation, and in fatty acid composition.

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Studies on Sulphatases

25. THE DETERMINATION OF $BaS^{16}O_3{}^{18}O$ BY INFRARED SPECTROSCOPY*

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In vitro, the various enzymes of the sulphatase group hydrolyse their specific sulphate ester substrates with the formation of inorganic sulphate and the corresponding desulphated hydroxy compound. Whether this is also their action *in vivo* or whether they can act as transferases is not yet known with certainty. Two transferring activities, involving the transfer of either the sulphate group or the desulphated residue, can be envisaged. A knowledge of the position of cleavage of the sulphate esters by

* Part 24: Lloyd (1959).