phage is inactivated logarithmically with time over a wide temperature range whereas the wet preparations of phage are not.

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Phospholipids of Human Red Blood Cells.* (24670)

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The wide disagreement in reported values for concentrations of individual phospholipids in human red blood cells(1-5) indicates inadequacy of methods employed. Recently, it was shown that the main phospholipids in serum lipid extracts could be isolated and quantitated by chromatography on silicic acid(6,7). Concentrations of individual serum phospholipids in these studies were at variance with many previously reported values. In the present study, the same chromatographic method was used to isolate and quantitate the main phospholipids of red blood cells of fasting normal human subjects. The main components found were ethanolamine- and serine-containing phospholipid, lecithin, sphingomyelin, and lysolecithin. Concentrations of these components differed from those of several previously reported series(1-4).

Materials and methods. Blood was obtained from 3 male and 3 female apparently healthy young white adults in fasting state and collected in test tube containing heparin. It was centrifuged and plasma and buffy coat removed. Red blood cells were washed 3 times with equal volume of isotonic saline, then packed by centrifugation at 3500 rpm for 30 minutes. One volume of packed red blood cells was pipetted from bottom of tube and hemolyzed with equal volume of water (8). Extraction of this hemolysate was accomplished according to the method of Folch *et al.*(9). The hemolysate was added with shaking to 20 volumes (per volume of packed red blood cells) of chloroform-methanol (2:1, v/v), let stand 30 minutes, and filtered. The red blood cell precipitate was dried, ground to powder, and reextracted with 10 volumes (per volume of packed red blood cells) of chloroform-methanol (2:1, v/v) twice, in the same manner. The 3 filtrates were pooled and then emulsified with 0.2 its volume of 0.05 N KCl solution. The emulsion was broken by centrifugation at 2500 rpm for 15 minutes to give 2 distinct phases without fluff at the interface. The upper phase was removed and the lower phase taken to dryness in rotary vacuum evaporator at maximum temperature of 60°C. This dried extract was stored *in vacuo* at –30° C. Chromatography of the lipid extract on silicic acid columns and on silicic acid-impregnated filter paper and chromatography of acid hydrolysates of phospholipid fractions on filter paper have been described(6,7). Methods used in analyses of column fractions have also been reported(6, 7). In the present study, the standard procedure in normal subjects was to extract 5 ml of packed red blood cells and chromatograph 2 aliquots of the extract, equivalent to 2 ml of packed red blood cells each, on 2 1-g silicic acid columns, respectively.

Results. Three extractions of hemolyzed red blood cells were routinely performed,

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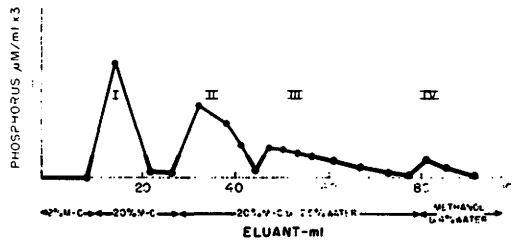

FIG. 1. Chromatography of red blood cell lipid extract on silicic acid (1 g). Predominant peak substances appeared to be: I, ethanolamine- and serine-containing phospholipid; II, lecithin; III, sphingomyelin; IV, lysolecithin.

since the first 2 extractions removed most of the lipid phosphorus, a third extraction about 7-9% in addition, and a fourth extraction about 1-2% in addition. The first 3 extractions, therefore, probably removed more than 95% of total phospholipid extractable under these conditions. Washing pooled extracts with 0.05 N KCl solution removed approximately 10-20% of the extracted phosphorus. This water-soluble phosphorus was assumed to be non-phospholipid phosphorus, as (1) it was insoluble in chloroform and (2) in the test for phosphorus(10) it produced a blue color of similar intensity with or without prior digestion. Phosphorus in the chloroform phase was probably almost all, if not all, present as phospholipid, since (1) in the test for phosphorus it did not react prior to digestion and (2) about 95% of it had the mobility of phospholipid on the silicic acid column.

Chromatography of the lipid extract on silicic acid columns gave rise to 4 peaks of phosphorus concentration (Fig. 1) with mobilities, in order of decreasing Rf, of the "cephalin", lecithin, sphingomyelin and lysolecithin, respectively, of lipid extracts of serum(7). Chromatography of the lipid extract on silicic acid-impregnated filter paper similarly gave rise to 4 spots, the fastest staining with the ninhydrin method, and the 3 slower with the phosphomolybdic acid method. The slowest and faintest spot was streaked down from the origin. Two spots stained with Schiff's stain: (1) the ninhydrin-reacting spot and (2) the fastest spot staining with the phosphomolybdic acid method, the former staining much more intensely than the latter. These 4 spots had mobilities, in order of decreasing Rf, similar to those of the "cephalin", lecithin, sphingomyelin and lysolecithin, respectively, of lipid extracts of serum(7). Hydrolysis of peak materials with 6N HCl at 110°C for 18 hours in a sealed glass tube followed by chromatography on filter paper with butanol-acetic acid-water (4:1:4, using butanol layer) elution showed that the first peak material gave rise to 2 main ninhydrin-reacting spots with mobilities of ethanolamine and serine, respectively, and lesser ninhydrin-reacting spots, but no spot staining with the phosphomolybdic acid method, while the second, third, and fourth peak materials each gave rise to 1 spot staining with the phosphomolybdic acid method, with the mobility of choline, and 2 or 3 minor ninhydrin-reacting constituents. The third peak material (sphingomyelin), in addition, produced a second spot staining with the phosphomolybdic acid method but with an Rf greater than that of choline; a spot with similar mobility and concentration in proportion to the choline spot was seen with the hydrolysate of the third peak material of serum(7). This spot remains unidentified. Phosphorycholine,† a possible hydrolytic product of sphingomyelin, did not stain with the phosphomolybdic acid method.

When these acid hydrolysates of peak materials were chromatographed on filter paper using acetone-water (4:1) elution, a spot with the mobility and staining properties of inositol was found only with the first peak material. This inositol, however, was estimated to comprise on a molar basis less than 1% of total red blood cell phospholipids.

The last peak materials of 2 samples were rechromatographed on the silicic acid column using the same solvent system as for the lipid extract. The phosphorus redistributed approximately as follows: "cephalin" 12%, lecithin 16%, sphingomyelin 22%, and lysolecithin 50%. It was assumed, therefore, that about one-half of the last peak material was lysolecithin, and in calculating the relative distribution of individual phospholipids, the other half was added to the other 3 fractions

† Obtained from Mann Research Labs., N. Y., as barium phosphocholine chloride. Sodium salt dissolved in 0.1 N HCl and applied to filter paper likewise did not stain.

TABLE I. Duplicate Analyses of Red Blood Cell Phospholipids of 6 Normal Subjects.

Sex	Age	% total lipid P				% recovery of P added to column	μMoles lipid P/ml RBC
		"Cephalin"*	Lecithin†	Sphingomyelin	Lysolecithin‡		
♂	23	43.0	33.5	21.8	1.7	99	4.80
		42.7	34.7	20.7	1.9	97	
♂	24	42.5	30.0	26.0	1.5	97	4.41
		42.4	29.3	26.7	1.6	99	
♂	30	41.2	36.0	20.5	2.3	101	4.77
		41.7	38.3	18.0	2.1	95	
♀	26	44.6	33.3	20.3	1.8	88	4.44
		41.0	33.1	23.9	2.0	94	
♀	30	40.7	34.3	23.1	1.9	97	5.11
		41.9	32.5	23.9	1.7	94	
♀	31	44.7	29.4	24.0	1.8	96	4.43
		43.8	29.9	24.3	2.0	97	
	Avg	42.5	32.9	22.8	1.9	96	4.66

* Includes plasmalogen and inositol-containing phospholipid.
† Includes plasmalogen.
‡ Estimated as being 50% of Peak IV; 12% of Peak IV added to "cephalin," 16% to lecithin, and 22% to sphingomyelin (see text).

according to the above percentages. The last peak material, both before and after rechromatography, was hemolytic when incubated for 2 hours at 37°C with 2 ml of a 1% suspension of washed sheep red blood cells suspended in isotonic saline; 0.05 μM of the rechromatographed material produced moderate hemolysis (0.01 μM of the corresponding lysolecithin fraction obtained from serum produced barely visible hemolysis(6)). The molar ester bond-to-phosphorus ratio was 1.1 in the one sample analyzed.

The average molar ester bond-to-phosphorus ratio of the "cephalin" peak in the 4 samples analyzed was only 1.2, while this ratio in the one sample of the lecithin peak analyzed was 1.5. A similar ratio in the lecithin peak was found in serum(7).

Analyses for concentrations of individual phospholipids were carried out in duplicate on red blood cells of 6 apparently normal adults in the fasting state (Table I). The valley between lecithin and sphingomyelin peaks was usually not as deep as depicted in Fig. 1; the valley tube was calculated as containing one-half lecithin and one-half sphingomyelin(7).

Discussion. The values found for relative concentrations of individual serum phospholipids were at variance with results obtained by others(1-4) and agreed best with those reported by Hack(4). Previous reports have shown considerable variation between normal subjects in the same series, as well as between series of different investigators(1-4), suggesting inadequacies in methods employed. In contrast, the relatively good agreement between duplicate samples and the rather small variation between subjects in our study provide evidence for reproducibility of the method. The present method, moreover, seemed relatively simpler than previous methods and was carried out on only 2 ml aliquots of packed red blood cells, although a considerably smaller quantity could have been used. At least as much lipid phosphorus appeared to be extracted as with previous methods.

The phospholipid fractions separated in our study were to some extent mixtures. The "cephalin" fraction was composed largely of ethanolamine- and serine-containing phospholipid; in addition, there was evidence for plasmalogen, a small amount of inositol-containing phospholipid, and unidentified ninhydrin-reacting components. Axelrod *et al.*(5) estimated that of total red blood cell phospholipids, 23% was phosphatidyl ethanolamine and 15% phosphatidyl serine. The lecithin fraction apparently contained some plasmalogen and the sphingomyelin fraction an unidentified phospholipid, evidence for which was also found in the sphingomyelin

fraction of serum lipid extract(7). The lysolecithin fraction contained material from the other 3 fractions; from rechromatography studies, it was estimated to contain about 50% lysolecithin. Hydrolysates of all fractions contained small amounts of ninhydrin-reacting substances which may have represented constituents of as yet unidentified phospholipids. The low molar ester bond-to-phosphorus ratios of the "cephalin" and lecithin fractions, as was observed with serum (7), may be related to a defect in the ester bond method, to alteration of phospholipids during isolation procedure, or to presence in these fractions of other phospholipids, such as plasmalogen, with ester bond-to-phosphorus ratios of less than 2.

No previous investigators reported the presence of lysolecithin, which comprised about 2% of total red blood cell phospholipids. It is unlikely that this represented adsorbed serum lysolecithin, as the red blood cells were washed 3 times with isotonic saline before extraction, or that it was produced as an artifact during the extraction or isolation procedure(6).

It is of interest to note the striking contrast in the relative proportions of individual phospholipids in red blood cells as compared to serum, especially since there is evidence for exchange of phospholipids between these 2 compartments(11,12).

Summary. Human red blood cells were analyzed for individual phospholipids using chromatography on silicic acid. Concentrations of the main components, which appeared to be ethanolamine- and serine-containing phospholipid, lecithin, sphingomyelin, and lysolecithin, were measured in red blood cells of 6 normal fasting subjects.

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Effect of Epinephrine upon Plasma Optical Density, Unesterified Fatty Acids, Lipemia Clearing and Heparin Levels. (24671)

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The role of epinephrine in the physiology of fat mobilization has been known, and experimental evidence has been summarized(1,2). Importance of plasma unesterified fatty acids as the major fat transport mechanism involved in this effect of epinephrine was recently revealed(3,4). Subsequently tissue studies indicated that there was increased triglyceride lipolysis in fat depots following adrenalin injection(5), and that unesterified fatty acids were released(6). In these studies no observations were made of plasma lipemia clearing activity or of heparin levels. Since heparin lipoprotein lipase system probably plays a physiologic role in removal of alimentary lipemia from the bloodstream, and perhaps in tissue triglyceride lipolysis, it seemed desirable to study this mechanism in plasma following injection of epinephrine.

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Procedures and methods. The hormone was administered intravenously during post-absorptive period, in 9 dogs and 4 human volunteers. The former received .1 - .2 ml of 1:1000