PHOSPHOLIPIDS OF HUMAN HAIR LIPIDS

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INTRODUCTION

The composition of phospholipids in human sebum or scalp and hair lipids has not been reported to date. Recently, mixtures of such components were isolated from hair lipids by TLC over silica gel[1] and in the present study, procedures are advanced for the separation and analysis of the individual phospholipids. The saturated and olefinic acids obtained on hydrolysis of these components were analyzed by gas chromatography employing polar and nonpolar packings.

MATERIALS AND METHODS

Lipids were extracted with petroleum ether (b.p. 30–60°) from the hair of Negro men who abstained from the use of hair dressings and pomades. The hair was cut with degreased scissors exclusively, 5–7 days after shampooing with bar soap and all glassware and relevant equipment were degreased with petroleum ether and ethyl ether before use. The general procedures as described earlier were followed in all respects[2,3]. Phospholipids employed as standards were isolated from soy bean and brain and several were purchased from Sigma Chemical Co. and Nutritional Biochemicals Corp. Purity of the samples was also checked by analysis of phosphorus[4] and nitrogen[5]. Fatty acid standards were secured from Applied Science Laboratories, Inc. and several highly unsaturated types originated from special collections.

For the isolation of the phospholipid mixtures, the hair lipid sample (CB-29) was submitted to TLC separation on Silica Gel G, the developing medium being 98 % acetone + 2 % petroleum ether by volume as presented previously[1]. The first fraction of low mobility which was eluted from the gel portion with chloroform–methanol, contained the phospholipids and amounted to 8.0 % of the starting lipids.

TLC separation of phospholipids

Glass plates of 20 × 20 × 0.4 cm were coated with Silica Gel G at a thickness of 0.25 mm and air-dried at 25° for 30 min. They were cooled and stored over silica gel until use. An aliquot of sample in amount of 5–8 μg dissolved in absolute methanol was applied at the corner, 2.0 cm from the lower edge and the chromatoplate dried in a stream of nitrogen. Two glass chambers containing solvents I (chloroform–methanol–6 *N* ammonium hydroxide, 65:30:5) and II (chloroform–methanol–6 *N* ammonium hydroxide, 30:65:5), respectively, were equilibrated for at least 2 h prior to the run. Ascending development was carried out with medium I for 1 h, the plate then being

removed from the chamber, the solvent front marked and the plate dried in an atmosphere of nitrogen. For two-dimensional TLC, the chromatoplate was introduced into the chamber with medium II for 1 h and dried in nitrogen. The plates could be sprayed with a variety of detection reagents. Reference standards were also run simultaneously, the gel portions being scraped off, pooled separately and each eluted exhaustively with chloroform, 1:1 chloroform–methanol and then methanol.

Hydrolysis of phospholipids

Small samples were heated in sealed tubes with 1.7 *N* methanolic hydrochloric acid for 4 h at 100° by the procedure of DAWSON[6]. The hydrolysates were taken to dryness under reduced pressure at 90°. Water was added to each ampule and a portion of the sample was submitted to TLC. For isolation of the fatty acids, each of the phospholipids in methanol was refluxed with 1 *N* sodium hydroxide for 3 h. The mixture was cooled, acidified with 30 % sulfuric acid and the fatty acids extracted with ether. The respective methyl esters were prepared by addition of diazomethane. As sphingomyelin is resistant to alkaline treatment, hydrolysis and esterification were carried out with 5 % anhydrous methanolic hydrochloric acid[7].

Gas chromatography

A Barber Colman model 5000 gas chromatograph with a hydrogen flame detector was used in the analysis of fatty acid esters. The U-shaped borosilicate column measured 8 ft. × 1/4 in. O.D. and contained 3 % SE-30 on 60-80 mesh Gas Chrom P. The column, injector and detector temperatures were 250°, 270° and 300°, respectively; He was the carrier gas at 70 ml/min. With the system containing 15 % DEGS on 80–100 mesh Gas Chrom P, the column, injector and detector temperatures were 200°, 220° and 250° in the order stated; He was introduced at 65 ml/min. The samples were dissolved in ether and volumes of 5 μl injected. GC analysis was performed directly on the ester mixtures as such and on aliquots hydrogenated in a Parr low pressure apparatus at 25° in the presence of platinum oxide catalyst. Tentative assignments for each peak were obtained from a semilogarithmic plot of relative retention time *vs.* chain length and degree of unsaturation of standard mixtures of fatty acid methyl esters.

Separation of saturated and unsaturated acids by TLC

As an adjunct to the above procedures, preliminary separation of the fatty acids was effected by TLC. Chromatoplates containing silica gel were prepared as described and the mixtures of fatty acids in ether applied, the developing medium being 70 % ethanol saturated with silver nitrate. The saturated acids remained at the point of application while the unsaturated components migrated.

RESULTS

Isolation of phospholipids by TLC

Several developing media were explored for the separation of phospholipids from the human hair source by TLC. R_F values are given in Table I for six solvents and of these, media I and II containing chloroform–methanol–6 *N* ammonium hydroxide were adopted for two-dimensional fractionation. The 8 phosphorus-

TABLE I

R_F VALUES OF PHOSPHOLIPIDS SUBMITTED TO TLC ON SILICA GEL G

Component	*Developing solvent*[a]					
	I	*II*	*III*	*IV*	*V*	*VI*
Glycerophosphorylcholine[b]	0.98	0.97				
Phosphatidic acid	0.85	0.84				
Phosphatidylethanolamine	0.70	0.74	0.08	0.40	0.02	0.71
Phosphatidylcholine	0.47	0.37	0.00	0.02	0.00	0.45
Phosphatidylinositol	0.43	0.71				
Phosphatidylserine	0.31	0.57	0.35	0.02	0.01	0.10
Sphingomyelin	0.31	0.33	0.00	0.03	0.00	0.05
Lysophosphatidylcholine	0.23	0.26	0.24	0.00	0.00	0.39

[a] The compositions of the developing media on a volume basis were as follows: I = chloroform–methanol–6 *N* ammonium hydroxide (65:30:5); II = chloroform–methanol–6 *N* ammonium hydroxide (30:65:5); III = 60% methanol; IV = butanol saturated with water; V = anhydrous isopropyl alcohol; VI = 70% ethanol.

[b] For choline, the R_F values with solvents III–VI, inclusive, were 0.70, 0.20, 0.26 and 0.80, respectively.

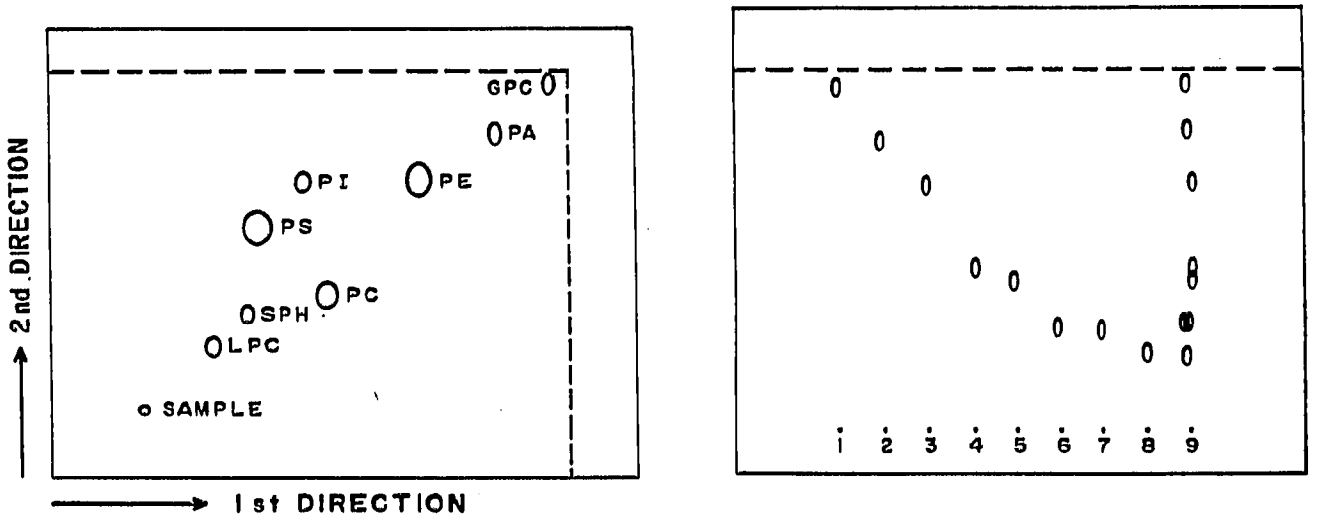

Fig. 1. Thin-layer chromatogram of the phospholipids from hair lipids. The developing solvent in the first direction was chloroform–methanol–6 *N* ammonium hydroxide (65:30:5) and in the second, the ratio of the same solvents was 30:65:5. The dotted lines indicate the solvent fronts. O = Origin; LPC = lysophosphatidylcholine; SPH = sphingomyelin; PC = phosphatidylcholine; PS = phosphatidylserine; PI = phosphatidylinositol; PE = phosphatidylethanolamine; PA = phosphatidic acid; GPC = glycerophosphorylcholine.

Fig. 2. Thin-layer chromatograms of the various phosphatides obtained on rechromatography of the initial TLC fractions and developed with solvent I (chloroform–methanol–6 *N* ammonium hydroxide; 65:30:5). Spot 9 denotes the mixture of phospholipids and the other spots represent the following: 1 = glycerophosphorylcholine; 2 = phosphatidic acid; 3 = phosphatidylethanolamine; 4 = phosphatidylcholine; 5 = phosphatidylinositol; 6 = phosphatidylserine; 7 = sphingomyelin; 8 = lysophosphatidylcholine.

containing spots as obtained from the starting mixture by this method are shown in Fig. 1. The individual components extracted from the respective gel portions on rechromatography over Silica Gel G and employing solvent I as developer, yielded the spots as indicated in Fig. 2 and which coincided in R_F with authentic samples or standards run concurrently. The various spots were located by color formation with sprayed reagents. Molybdenum types[8,9], iodine[10] and Schiff's reagent[11] were positive for each of the 8 components; color development occurred on treatment of glycerophosphorylcholine and phosphatidylinositol with silver nitrate[12] and of glycerophosphorylcholine, phosphatidylcholine,sphingomyelin and lysophosphatidylcholine with bismuth trichloride[13]. The phosphatidylethanolamine and phosphatidylserine portions were stained by ninhydrin spray.

Identification of hydrolytic products

Further substantiation of the 8 components obtained above was afforded by their hydrolysis and TLC resolution. Color reactions and R_F were also compared with reference standards chromatographed simultaneously. Methanol–water–6 *N* ammonium hydroxide (60:30:10) was employed in conjunction with serine, ethanolamine and choline and isopropyl alcohol–acetic acid–water (60:20:20) for development of inositol, glycerol and phosphatidic acid (Fig. 3).

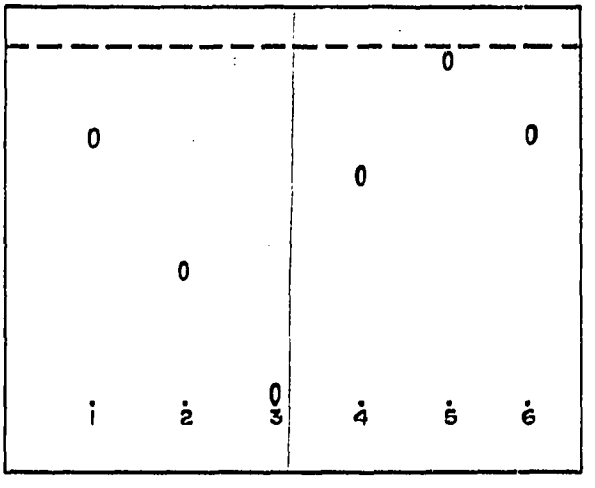

Fig. 3. TLC analysis of hydrolytic products from human hair phospholipids. The developing medium for spots 1–3, inclusive, was methanol–water–6 *N* ammonium hydroxide (60:30:10) and for 4–6, inclusive, isopropyl alcohol–acetic acid–water (60:20:20). Spot 1 = serine; 2 = ethanolamine and phosphorylcholine as both have almost the same R_F value; 3 = choline; 4 = inositol; 5 = phosphatidic acid; 6 = glycerol. Reference standards and color developing reagents were also employed for identification.

Composition of phospholipids and derived fractions

The distribution of the individual phospholipids is presented in Table II. An attempt was also made to resolve the initial mixture into fractions by dropwise addition of ethanol with constant stirring to an ethereal solution of the phospholipids until complete precipitation of "cephalin" occurred. "Cephalin" and "lecithin" amounted to 30.2 % and 69.6 %, respectively, on a weight basis. The eight components were present in the "lecithin" fraction at the levels designated in Table II, whereas, "cephalin" contained solely phosphatidylethanolamine and phosphatidylserine.

TABLE II

PHOSPHOLIPID COMPOSITION OF HUMAN HAIR LIPIDS (CB-29) AND FRACTIONS

Component	*Initial phospholipid mixture* (%)	*Lecithin* (%)	*Cephalin* (%)[a]
Glycerophosphorylcholine	5.0	6.0	
Phosphatidic acid	7.8	9.3	
Phosphatidylethanolamine	20.6	20.8	21.6
Phosphatidylcholine	13.3	15.6	
Phosphatidylinositol	8.1	9.9	
Phosphatidylserine	30.1	20.5	78.4
Sphingomyelin	7.0	8.0	
Lysophosphatidylcholine	8.1	9.9	

[a] Separated from the initial phospholipid mixture by ethanol addition to an ethereal solution.

The distribution of phospholipids was also checked by use of the Florisil method for the separation of neutral lipids advanced by CARROLL[14]. As the column was washed with moderate amounts of ether after elution with 4 % acetic acid in ether and just prior to the passage of methanol, so as to remove excess acid, the yields were somewhat lower as compared to the TLC procedure. In one experiment employing 500 mg of CF-29 in a 20 × 1.5 cm column charged with 20 g Florisil, the recovery of phospholipids in the methanol eluate was 34 mg or 6.8 %; its composition simulated the one given in Table II.

Fatty acid composition of the phospholipids

The fatty acids obtained on hydrolysis of the phospholipids were converted to the methyl esters and analyzed by gas chromatography as described above. Table III presents the tentative identification based on the relative or area percent for lysophosphatidylcholine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin and phosphatidylethanolamine.

DISCUSSION

The separation of phosphatides from lipids extracted from human hair cuttings has been accomplished by TLC and the individual components fractionated by further TLC procedures (Table II). As based on the classical gravimetric approach, the lecithin fraction (69.6 %) contained 8 phosphatides and the cephalins (30.2 %) constituted phosphatidylethanolamine and phosphatidylserine in the ratio of 3.7:1. Glycerophosphorylcholine, lysophosphatidylcholine and phosphatidic acid occur as a result of some hydrolysis. In relation to the starting hair lipids (CB-29), the overall weight percentages of the eight components isolated were 0.3–0.5, except for levels of 0.9, 1.4 and 2.0 for phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, respectively.

The hydrolysis of the individual phosphatides led to mixtures of fatty acids which were analyzed by gas chromatography. For check purposes, TLC was employed in conjunction with several products, the developing medium being 70 % ethanol

TABLE III

RELATIVE PERCENTAGE DISTRIBUTION OF FATTY ACIDS IN HAIR LIPID PHOSPHATIDES

Fatty acid[a]	*Lyso-phosphati-dylcholine*	*Phosphati-dylcholine*	*Sphingo-myelin*	*Phosphati-dylinositol*	*Phosphati-dylserine*	*Phosphatidyl-ethanolamine*
13:0		2.0			1.3	2.0
14:0	9.0	9.3	7.8	5.7	6.7	6.6
14:1	1.0	1.2		0.4		1.2
15:0	2.9	2.4	5.3	2.3	6.7	2.3
15:1		0.6				
16:0	37.5	24.5	34.9	29.6	17.5	20.4
16:1	7.2	12.1	4.3	10.5	1.3	11.8
17:0	2.0	0.6	2.3		2.7	
17:1		0.7	2.2	0.5	2.7	1.1
18:0	13.1	4.7	5.5	7.1	16.2	9.5
18:1	17.1	14.5	7.4	20.1	12.2	12.1
18:2	3.4	4.1	7.0	4.0	6.8	3.3
19:0		1.0	2.5			
20:0			1.7			
20:1	1.5	1.7		2.1	4.0	4.6
20:2	2.3	0.5		1.9	1.4	
20:3	0.1	0.3				1.6
22:0	0.1		3.8		2.7	
20:4	0.2	2.1	3.9	3.6	2.7	9.8
23:0			3.2			
22:1	1.2	1.8		1.0	4.0	3.5
22:2	0.2	0.3		0.9	2.7	
22:3		1.3	1.3			
22:4	0.4	8.3		1.8	1.4	3.2
24:0	0.2	1.2	3.7	2.3	1.3	0.5
24:1		2.1	1.8	4.0	2.7	2.3
22:5		1.3		0.4	1.4	0.9
22:6		1.3	0.9	1.2	1.3	2.9

[a] Designated by the number of carbon atoms: number of double bonds.

saturated with silver nitrate; the unsaturated acids migrated from the point of application. As discussed previously in regard to alcohols of hair lipids[15], this method circumvents the darkening of plates observed with chromatoplates impregnated with the silver salt.

Analysis of the fatty acids from each of the 6 phosphatides showed the presence of moderate to high levels of the following: 14:0 (5.7–9.3 %), 16:0 (17.5–37.5 %), 16:1 (4.3–12.1 %; low in phosphatidylserine which contained 1.3 %), 18:0 (4.7–16.2 %), 18:1 (7.4–17.1 %) and 18:2 (3.3–7.0 %). Several of the fatty acids were not detected in each of the mixtures. Thus, the 15:1 component occurred solely in phosphatidylcholine, 19:0 and 22:3 in the latter as well as in sphingomyelin and 20:0 in the last mixture. Sphingomyelin also lacked the C_{20}-components, 22:4 and 22:5. The fatty acid data are further summarized in Table IV.

Lysophosphatidylcholine contained the lowest level of polyunsaturated acids (6.6 %) and of the olefinic components, odd-carbon acids could not be detected. In contrast to phosphatidylcholine, aside from lacking several of the unsaturated acids, the lysophosphatidylcholine ranged higher in both hexadecanoic and octadecanoic acids and the average number of double bonds/molecule was 0.5 or half that of the

phosphatidylcholine. The latter contained 8.3 % of the 22:4 acid as compared to 0.2 % for the lysolecithin. The fatty acid mixture from phosphatidylinositol paralleled that of phosphatidylcholine in average double bond number but contained higher levels of both hexadecanoic and octadecenoic acids.

For the cephalin components, the hexadecanoic and octadecenoic acid contents were somewhat diminished as compared to the above phosphatides. The overall unsaturated acid level of phosphatidylethanolamine (58.3 %) exceeded that of phosphatidylserine (44.6 %), the mean double bond numbers per molecule being 0.8 and 1.3, respectively.

TABLE IV

SUMMARY OF PERCENTAGE FATTY ACID DISTRIBUTION IN PHOSPHOLIPIDS[a]

Phosphatide	*Acids*								
	Unsaturated		*Odd-carbon*		*Even-carbon*		*Total content*		*Double bonds per molecule*
	Mono-	*Poly-*	*S*	*U*	*S*	*U*	*S*	*U*	
Lysophosphatidylcholine	28.0	6.6	4.9	—	59.7	34.6	64.6	34.6	0.5
Phosphatidylcholine	34.7	19.5	6.0	1.3	39.7	52.9	45.7	54.2	1.0
Sphingomyelin	15.7	13.1	13.3	2.2	57.4	26.6	70.7	28.8	0.6
Phosphatidylinositol	38.6	13.8	2.3	0.5	44.7	51.9	47.0	52.4	0.9
Phosphatidylserine	26.9	17.7	10.7	2.7	44.4	41.9	55.1	44.6	0.8
Phosphatidylethanolamine	36.6	21.7	4.3	1.1	37.0	57.2	41.3	58.3	1.3

[a] Based on the data of Table III; S and U refer to saturated and unsaturated, respectively.

Of the mixtures, sphingomyelin displayed the highest saturated fatty acid content (70.7 %) and of which, hexadecanoic acid (34.9 %) was most prominent; the level of octadecenoic acid (7.4 %) was lowest of the groups. It simulated lysophosphatidylcholine in the ratio of overall saturated to unsaturated components and the mean double bond number was 0.6/molecule. Also, in marked contrast to the other five, the ratio of mono- to polyunsaturated acids was almost unity.

The phosphatides of human hair lipids are unique in composition when compared to those of other sources. CARRUTHERS[16] analyzed epidermal phospholipids from normal mice and those displaying malignant transformations and noted definite differences among the animal groups as well as between mouse and human epidermal lecithins. Such compositions are in marked contrast to those advanced in the present report.

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SUMMARY

Thin-layer chromatography has been applied to the separation of a mixture of phospholipids from human hair lipids and to its resolution into 8 components. The

latter consisted of glycerophosphorylcholine, phosphatidic acid, lysophosphatidylcholine, phosphatidylinositol, phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine; the last three phosphatides made up 65 % of the mixture. The purified phospholipids were submitted individually to hydrolysis and the respective fatty acid esters analyzed by gas chromatography. Sphingomyelin contained the highest level of saturated acids and the ratio of mono- to polyunsaturated acids was close to 1. Phosphatidylethanolamine acids ranged high in unsaturated members and displayed an average double bond number of 1.3/molecule.

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