

# THE PHOSPHATIDES OF HUMAN PLASMA. II. ABNORMALITIES ENCOUNTERED IN THE NEPHROTIC SYNDROME \*

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Abnormalities in the plasma lipids have been correlated with the nephrotic syndrome for many years. Zone (3) and immunoelectrophoresis (4), Cohn fractionation (5), and ultracentrifugal analysis (6, 7) have all confirmed increased concentrations of certain plasma lipoproteins. Recent studies by Baxter, Goodman and Havel (7) have documented abnormal proportions of cholesterol, triglyceride and phospholipid in the lipoprotein classes.

Neither the course of events leading to these abnormalities nor their role in the manifestations of the nephrotic syndrome is well understood. Data on the phosphatides in this condition have been limited to alterations in the levels of the lipid phosphorus in the plasma and in the lipoprotein classes (7), and to changes in the incorporation of  $P^{32}$  into the lipid phosphorus of plasma (8). A change in the composition of the plasma phosphatides has been postulated on the basis of these  $P^{32}$  experiments. This study was undertaken to investigate, by the use of chromatographic techniques, the individual phosphatides in the plasma of patients with the nephrotic syndrome.

## METHODS

Hospitalized patients with the nephrotic syndrome were studied on the medical and pediatric wards of Strong Memorial Hospital. Venipuncture was performed in the fasting state (except for plasmas no. 7, 18 and 21, Tables I and II, which were obtained immediately after light lunches) and blood was collected in a bottle containing potassium oxalate. Plasma separation, lipid extraction, and quantitative paper and column chromatography were performed as previously described (9). Serum cholesterol and serum albumin were determined in the Clinical Chemistry Laboratory of the University of Rochester Medical Center by the method of Bloor (10)

\* Some of the data have been reported in abstract form (1, 2).

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and by electrophoresis with a Spinco apparatus at pH 8.6, respectively.

Preparative ultracentrifugation of plasma was carried out as described by Havel, Eder and Bragdon (6). Plasma densities were adjusted with solutions of KBr and NaCl to yield the following density (D) classes:  $<1.019$ ,  $<1.063$ , and  $>1.063$ . The components of the class D 1.019–1.063 were calculated by difference whenever feasible. Separation was accomplished in a Spinco model L ultracentrifuge with a 40 rotor, with 105,400 G at the tube centers for 22 hours at 2° C. The supernatant layer of each tube was removed by pipet and was transferred to a 100 ml extraction tube. Lipid adhering to the tube cap was washed into the extraction tube with 0.15 N saline solution. After the entire supernatant layer and the beginning of the clear intermediate zone had been transferred, the plastic ultracentrifuge tube was cut with scissors and the upper portion of it was added to the extraction tube. The bottom of the ultracentrifuge tube, containing the infranate and the remainder of the clear zone, was placed in a second extraction tube. Lipid extraction was performed with 29 ml methanol-chloroform 25:4 (vol/vol), followed by 15 ml methanol-chloroform 1:1 used three times.

The first three extractions were at 60° C for 2 minutes each, and the fourth extraction was for 1 hour at 60° C. After adding benzene, the pooled extracts were taken to dryness with a rotary vacuum evaporator. The lipids were re-extracted with chloroform, leaving behind a large amount of residue, presumably composed mostly of KBr and perhaps some NaCl. Further preparation of each extract, and phosphatide analysis by quantitative paper chromatography were carried out as previously described (9).

While lipid extracts of whole plasma have always dried to a constant weight of 60° C under a stream of nitrogen in less than 1 hour, and have then appeared solid, extracts of the density classes have never become more than partially solidified after 1 hour of drying, at which time isoamyl alcohol-benzene 1:1 was added, and chromatography was carried out.

## RESULTS

*Distribution of major phosphatides in whole plasma.* Table I shows the data on plasma samples obtained from each patient during the study, and Table II shows the data on serial plasma sam-

TABLE I  
Data on plasma samples of all patients with the nephrotic syndrome

Plasma	Patient	Chloro- form- soluble P	Total lipid	Appearance of plasma	Serum choles- terol	Serum albumin	Lyso- lecithin	Sphingo- myelin	Lecithin	Phosphatide phosphorus		
		$\mu\text{g/ml}$	$\text{mg/ml}$		$\text{mg/100 ml}$	$\text{g/100 ml}$	$\text{P/ml plasma}$			Lyso- lecithin	Sphingo- myelin	Lecithin
										%	%	%
Group A												
1	F.G.	154	16.56	clear	365		26.9	32.5	45.2	25.8	31.1	43.2
2	E.J.	119	12.6	clear	452	1.4	16.5	27.4	20.6	25.6	42.5	32.0
3	J.S.	135	14.75	clear	522		6.1	21.2	7.4	17.6	61.1	21.3
4	D.C.	82	11.3	clear	468	1.4	10.7	20.1	37.4	15.7	29.5	54.8
5	M.D.	173	21.3	mod. lact.	620	2.4	20.6	39.3	93.6	13.4	25.6	61.0
6	G.C.	118	13.5	clear	420	1.1	26.1	29.5	60.0	22.6	25.5	51.9
7	M.L.	83	14.8	lact.	564	2.4	17.8	25.1	16.6	29.9	42.1	28.0
8	B.W.	217	28.2	lact.	890	0.6	27.4	54.0	110.0	14.3	28.2	57.5
9	H.C.	187	20.6	slightly lact.	880	2.1	21.6	54.8	106.0	11.8	30.0	58.2
Group B												
10	L.A.	116	12.0	clear	213	1.1	10.3	26.2	56.8	11.0	28.1	60.9
11	B.W.	262	60.7	lact.			41.8	57.8	140.0	17.5	24.1	58.4
12	B.W.	169	49.7	lact.	1,734	1.5	22.5	38.2	105.2	13.6	23.0	63.4
13	L.C.	113	10.1	clear	375		15.4	26.1	67.2	14.2	24.0	61.8
14	L.A.	141	13.3	clear	432	1.1	6.7	30.0	67.1	6.4	28.9	64.7
Normal distribution												
15	L.C.	158	13.6	clear	400		10.6	31.6	107.0	7.1	21.1	71.8
16	J.S.	90	10.2	clear	364	2.5	7.9	16.9	46.1	11.1	23.8	65.1
Ranges of normal values												
							5.4- 9.5	12.4- 20.6	30.9- 71.7	6.9- 11.7	17.6- 24.3	64.0- 73.2

ples of two of these patients. In ten samples in which the level of cholesterol was elevated and that of albumin was depressed, the level of chloroform-soluble phosphorus was normal. The level of chloroform-soluble phosphorus was raised in all other plasmas.

Elevations in the level of one, two, or all three

individual major phosphatides were found, regardless of the levels of the chloroform-soluble phosphorus, in all but two plasmas. In the latter (nos. 16 and 22) depressed or normal, but not elevated, levels of one or more phosphatides were found. However, when the proportions among the major phosphatides were considered, a single pattern of

TABLE II  
Data on serial plasma samples of two patients with the nephrotic syndrome

Plasma	Date	Chloro- form- soluble P	Total lipid	Appearance of plasma	Serum choles- terol	Serum albumin	Lyso- lecithin	Sphingo- myelin	Lecithin	Phosphatide phosphorus		
		$\mu\text{g/ml}$	$\text{mg/ml}$		$\text{mg/100 ml}$	$\text{g/100 ml}$	$\mu\text{g P/ml plasma}$			Lyso- lecithin	Sphingo- myelin	Lecithin
										%	%	%
Patient D.C.												
4	4/ 1/59	82	11.3	clear	468	1.4	10.7	20.1	37.4	15.7	29.5	54.8
17	8/31/59	125	17.3	clear	653	0.7	11.7	24.4	50.0	13.6	28.4	58.0
18	7/27/60	149	15.7	clear	641		12.4	43.8	92.8	8.3	29.4	62.3
19	8/13/60	121	11.1	clear	839		7.7	26.5	61.0	8.1	27.8	64.1
20	8/15/60	155	18.7	slightly lact.	905	1.6	10.4	38.3	80.8	8.0	29.6	62.4
21	8/22/60	163	11.7	clear	555	3.1	13.9	32.6	86.9	10.4	24.4	65.2
Patient E.J.												
2	2/ 3/59	119	12.6	clear	452	1.4	16.5	27.4	20.6	25.6	42.5	32.0
22	4/ 7/59	94	12.8	clear	600	1.1	4.6	15.7	41.0	7.5	25.6	66.9
23	4/11/59	137	13.0	clear	792	1.0	8.9	22.7	47.3	11.2	28.6	60.2
24	6/ 3/59	159	17.3	clear	455	0.6	11.8	37.0	91.4	8.4	26.4	65.2

TABLE III  
*Phosphatide analyses of lipoprotein density classes*

Plasma	Patient	Chloroform-soluble P	Density class	Lyso- lecithin	Sphingo- myelin	Lecithin	Phosphatide phosphorus		
							Lyso- lecithin	Sphingo- myelin	Lecithin
		$\mu\text{g/ml}$			$\mu\text{gP/ml plasma}$		%	%	%
6	G.C.	118	plasma	26.1	29.5	60.0	22.6	25.5	51.9
		17.1	<1.019	0.8	3.0	10.0	6.1	21.8	72.1
		48.8	<1.063	4.7	14.3	30.0	10.1	28.8	61.1
		29.7	>1.063	3.0	4.0	19.2	11.4	15.4	73.2
		31.7	1.019-1.063	3.8	11.3	20.0	11.0	32.1	56.9
9	H.C.	187	plasma	21.6	54.8	106.0	11.8	30.0	58.2
		106	<1.063	9.6	38.7	57.7	9.1	36.5	54.4
		28.8	>1.063	1.7	3.5	23.7	5.9	12.0	82.1
11	B.W.	262	plasma	41.8	57.8	140.0	17.5	24.1	58.4
		145	<1.063	18.9	26.3	82.5	14.8	20.6	64.6
		35.1	>1.063	1.9	4.1	23.8	6.4	13.6	80.0
	Normal subjects W.N.	94.3	plasma	6.7	15.5	53.0	8.9	20.6	70.5
		7.4	<1.019	0.59	1.73	4.73	8.4	24.6	67.0
		37	<1.063	2.52	11.1	23.8	6.7	29.7	63.6
		24.3	>1.063	3.28	2.8	17.2	14.1	12.0	73.9
		29.6	1.019-1.063	1.93	9.4	19.1	6.3	30.9	62.8
	W.N.*	88	plasma	11.9	15.1	49.8	15.5	19.7	64.8
		13.2	<1.019	1.0	2.9	8.1	8.1	24.3	67.6
		47.7	<1.063	3.6	9.0	30.7	8.3	20.8	70.9
		35.3	>1.063	6.0	3.9	22.7	18.3	12.0	69.7
		34.5	1.019-1.063	2.6	6.1	22.6	8.3	19.6	72.1

\* Sample obtained from nonfasting subject showing an increase in level and percentage of lysolecithin in plasma but lower values for these in D<1.063 than are shown by the abnormal plasmas.

abnormality was observed, consisting of an increase in the percentage of lysolecithin and sphingomyelin, and a decrease in the percentage of lecithin. For convenience, the samples have been separated into two groups, A and B. Under group A are listed those samples that showed the abnormalities mentioned above in all three major phosphatides, while under group B are listed the samples in which one or more of the major phosphatides were normal in proportion. Wherever abnormalities did occur in group B, they were in the same direction as those in group A. Although divided into two groups, the set of samples actually presented a spectrum of abnormality ranging in degree of severity from marked in some samples of group A, through minimal in many samples of group B, to two samples which had entirely normal proportions. The degree of abnormality in the proportions among the major phosphatides listed in Tables I and II was not related to the levels of total lipid, cholesterol, or chloroform-soluble phosphorus, or to the degree of lactescence of the plasma.

Ranges of normal values presented in the previous paper are listed at the bottom of Table I for comparison.

*Distribution of major phosphatides in lipoprotein classes.* Plasma samples 6 and 9 of group A, sample 11 of group B, and two normal samples were separated into density classes by ultracentrifugation. The phosphatide compositions of the density classes were determined in order to ascertain in which class the abnormal proportions occurred. The values obtained are listed in Table III.

The recovery of phosphorus in the density classes was relatively low, due probably to incomplete re-extraction of lipid by chloroform from the large amount of inorganic residue present in the methanol-chloroform extract of the density classes. Comparison of the levels of each phosphatide in the density classes with its level in plasma reveals that the loss of lecithin was least, while that of lysolecithin was greatest. In spite of this, the abnormal phosphatide relationship manifest in whole plasma still appeared in the

density classes, at  $D < 1.063$  in each instance, and in the class  $D 1.019-1.063$  when the contents of that class could be satisfactorily calculated.

*Minor phosphatide components.* The phosphatides of two group A plasmas and of three group B plasmas were also analyzed by combined quantitative column and paper chromatography. The results of these five analyses confirmed the presence of the abnormalities in proportions that had been found among the major phosphatides by quantitative paper chromatography. In addition, the minor phosphatides were analyzed. The sum of phosphatidylethanolamine plus lysophosphatidylethanolamine was determined in four samples and was present at the concentrations of 0.3 to 1.3  $\mu\text{g P}$  per ml plasma and constituted 1.1 to 2.4 per cent of the sum of the three major plus the three minor phosphatides. Inositol phosphatide was determined in three samples, was present at the concentration of 0.1 to 0.4  $\mu\text{g P}$  per ml plasma, and constituted 0.3 to 0.9 per cent of the sum of the six phosphatides. Values obtained for the three minor phosphatides of plasma from normal subjects and patients with several disease states, which were presented in the preceding paper (9), indicate that the concentrations of these phosphatides lie within relatively narrow ranges. Within these narrow ranges, the existence of significantly different means for the normal subjects and for patients with the nephrotic syndrome can be neither proven nor disproven until many more data are gathered. In all other samples, the minor

phosphatides have been investigated qualitatively. Inositol phosphatide has been detected in every sample by viewing the paper chromatogram under ultraviolet light after staining with rhodamine 6G. Lysophosphatidylethanolamine has been detected in many, but not all, samples and phosphatidylethanolamine has been detected in all but two samples (1 and 2) by a ninhydrin test on the paper chromatogram.

*Correlation of clinical findings with plasma phosphatide abnormalities.* Percutaneous renal biopsy was performed on all patients except M.L. and B.W. The biopsy findings and plasma phosphatide analyses are summarized in Table IV. All untreated subjects who have shown no glomerular change on biopsy and no clinical progression to chronic nephritis have had a group A phosphatide pattern (F.G., G.C., H.C., D.C.), whereas those patients who have had only a group B pattern have had evidence of underlying glomerular disease (L.A., L.C.). Transition from a group A to a group B pattern has occurred with successful steroid therapy (D.C.), with an indeterminate clinical course without steroid therapy (J.S.), and with progression to chronic nephritis (E.J.). Whether these patterns will be seen in future cases is conjectural.

#### DISCUSSION

Although many studies have been carried out, the etiology of the abnormalities seen in the circulating lipids in the nephrotic syndrome has not

TABLE IV  
*Correlation between phosphatide analyses and findings on percutaneous renal biopsy*

Plasma	Patient	Date	Phosphatide abnormality Group	Date of biopsy	Biopsy report*
1	F.G.	2/59	A	2/59	No glomerular change
6	G.C.	12/59	A	7/58	No glomerular change
9	H.C.	3/60	A	3/60	No glomerular change
5	M.D.	5/60	A	4/60	Membranous glom. change
4, 17	D.C.	4/59, 8/59	A	8/59, 8/60	No glomerular change
18, 19	D.C.	7/60, 8/60	B		
20, 21	D.C.	8/60, 8/60	B		
2	E.J.	2/59	A	2/59	Membranous glom. change
22, 23, 24	E.J.	4/59, 4/59, 6/59	B	9/59	Chronic glomeruloneph.
3	J.S.	12/58	A	11/59	Membranous glom. change
16	J.S.	9/59	B		
14	L.A.	6/59	B		
10	L.A.	8/59	B	8/59	Subacute glomeruloneph.
15	L.C.	8/59	B		
13	L.C.	9/59	B	10/59	Acute glomeruloneph.

\* All biopsies showed tubular changes compatible with nephrosis.

as yet been elucidated. Some investigators have felt that the increase in concentration of plasma lipids may be related to an inadequate "clearing mechanism," due chiefly to hypoalbuminemia, since, in subjects with the nephrotic syndrome, plasma lipid levels are reduced after intravenous infusions of albumin (11). However, quantitative changes in the production or removal of normally constituted circulating lipids seem inadequate to explain the phenomena that are observed. Since the increased concentration of lipids is apparently confined to the vascular compartment, and perhaps to the liver (12) and since the plasma lipids in the nephrotic syndrome can be influenced by certain factors which produce changes in them in normal subjects (13), the postulation of a "trapping effect" due to abnormalities in the plasma lipoproteins themselves has seemed reasonable. This was originally proposed by Byers, Friedman and Rosenman (14), and subsequently given experimental support by them (15).

There are two pieces of evidence which may be interpreted to mean that the lipoproteins of the serum in the nephrotic are abnormal. The first relates to the electrophoretic studies of Kunkel and Slater (3), and the second issues from the ultracentrifugal analyses of Baxter and colleagues (7).

Kunkel and Slater studied the lipoprotein pattern of hyperlipemic sera by zone electrophoresis. In childhood nephrosis they observed that  $\alpha$ -lipoprotein was markedly reduced,  $\beta$ -lipoprotein was absent, and that there were two or three irregular peaks between the  $\alpha$  and  $\beta$  positions. These peaks might have resulted from poor separation of the  $\alpha_2$  from the  $\beta$ -globulin (16), due partially to excess binding of free fatty acids to the  $\beta$ -lipoproteins (17), or they might have been partially due to abnormalities in the lipoproteins themselves. The ultracentrifugal analyses of Baxter and colleagues (7) have revealed abnormal proportions among the lipid constituents of individual lipoprotein fractions of nephrotic serum, which demonstrate that these lipoprotein fractions are not normal within themselves.

The marked disturbance in the nephrotic syndrome in the relationship among the plasma phosphatides reported in this paper, together with the finding that this is at least partially due to the similar disturbances within lipoprotein classes,

adds additional evidence that lipoproteins themselves are abnormal in this condition, and thus reinforces the basis upon which to build a theory of "trapping" to explain the development and maintenance of increases in the plasma lipid levels that are observed in the nephrotic syndrome.

If alterations in the rates of synthesis of individual phosphatides do occur, as is postulated, one may speculate on what metabolic changes might be responsible for this. The normal metabolic pathways for the individual phosphatides are not as yet completely worked out in experimental animals, and have not been extensively studied in human beings. Fishler, Entenman, Montgomery and Chaikoff (18) have demonstrated that  $P^{32}$ -inorganic phosphate is not converted to phospholipid in hepatectomized dogs, and that, when the liver is excluded from the circulation of the dog, the rate of disappearance of  $P^{32}$ -phospholipid is greatly prolonged. The livers of various animals have been found to contain all of the phosphatides that have been identified in human plasma (19). Enzyme systems have been prepared from liver that catalyze the formation of lecithin from diglyceride and cytidine diphosphate choline (20), of phosphatidylethanolamine from diglyceride and cytidine diphosphate ethanolamine (20) (both of these occur with the same enzyme), of sphingomyelin (in the form of a stereoisomer of the naturally occurring compound) from a ceramide and cytidine diphosphate choline (21), and of lecithin from lysolecithin (22).

Suppression of the conversion of lysolecithin to lecithin, and of diglyceride to lecithin and phosphatidylethanolamine, with diversion of phosphorylcholine to sphingomyelin, and of diglyceride to triglyceride, would make available to the plasma altered amounts of the phosphatides which would be compatible with the abnormal proportions found among the individual phosphatides in the plasma of patients with the nephrotic syndrome.

#### SUMMARY

The plasma phosphatides lysolecithin, sphingomyelin, and lecithin have been quantitatively analyzed by silicic acid chromatography in patients with the nephrotic syndrome, and an abnormality consisting of a higher proportion of lysolecithin and sphingomyelin, and a lower proportion of lecithin, has been found.

In the most markedly abnormal plasmas, all three of the above changes were found, while in the less abnormal plasmas, one or two of these changes were found, the remaining phosphatides being present in normal proportion. On fractionation of three abnormal plasmas by ultracentrifugation, an abnormality in phosphatide distribution was found in the low-density classes in each case. The degree of abnormality was not related to the levels of total lipid, cholesterol, or chloroform-soluble phosphorus, or to the serum albumin levels, nor could it be absolutely correlated with renal biopsy findings or with the clinical course in all cases. However, the demonstration of this abnormality in plasma and in lipoprotein classes is further evidence of the abnormal constitution of lipoproteins in the nephrotic syndrome.

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