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DETERMINATION OF CHOLESTEROL AND PHOSPHOLIPIDS OF HDL_2, HDL_3 AND VHDL IN PATIENTS WITH ATHEROSCLEROSIS AND DIABETES MELLITUS*)

By

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ABSTRACT

This report describes a method for and the results of an analysis of cholesterol and phospholipids in HDL_2 and HDL_3 and phospholipids in VHDL (d.>1. 210) of patients with cerebral thrombosis, myocardial infarction and diabetes mellitus.

After precipitation and elimination of LDL (low density lipoprotein) and VLDL (very low density lipoprotein) by Na phosphotangstate (NaPhT) and $MgCl_2$ and bringing the non-protein density to 1. 125, serum was subjected to ultracentrifugation with #494 angle rotor of IEC/B-60 ultracentrifuge (Kubota-International) at 105, 000xG for 22 hours.

The inadequate separation between HDL_2 and HDL_3 was observed at the clear zone beneath the top of fraction after centrifugation under these analytical conditions. Therefore, in this contaminated fraction, the HDL_2/HDL_3 ratio was calculated by the phospholipids/cholesterol ratio which had been substantially different between HDL_2 and HDL_3. VHDL-phospholipids was calculated by subtracting HDL_3-phospholipids from phospholipids of density higher than 1. 125.

The average HDL_2-cholesterol and phospholipids levels were 14. 8±4. 2 and 23. 9±5. 8 mg/dl respectively in eight healthy men and 18. 8±5. 0 and 28. 5±6. 8 mg/dl respectively in seven healthy women. The average HDL_3-cholesterol and phospholipid levels were 37. 6±7. 8 and 57. 3±11. 0 mg/dl respectively in eight healthy men and 40. 3±11. 9 and 64. 9±22. 1 mg/dl respectively in seven healthy women. The average VHDL-phospholipid level was 20. 4±5. 7 mg/dl in eight healthy men and 21. 4±4. 8 mg/dl in seven healthy women.

In patients with cerebral thrombosis, not only lipids in HDL_2 and HDL_3 but also VHDL-phospholipids significatly decreased. In patients with myocardial infarction, although lipids in HDL_2 and HDL_3 significantly decreased, VHDL-phospholipids maintained the normal value in most cases. In diabetic patients, HDL_2 decreased moderately but HDL_3 as largely as observed in patients with cerebral thrombosis and myocardial infarction, whereas VHDL-phospholipids were within the normal range.

Among four phospholipids in VHDL fraction, lecithin and lysolecithin were significantly lower in patients with cerebral thrombosis as compared with healthy subjects.

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INTRODUCTION

There has been suggested by a number of observations a strong inverse relation between the plasma levels of HDL (high density lipoprotein) and atherosclerosis in man[1,2]. The increased level of serum HDL protects humans from atherosclerosis and the dereased level predisposes them to it.

Determination of HDL-cholesterol is a convenient method that permits estimation of HDL in daily clinical examination and is in widespread use.

The HDL fraction isolated by the analytical centrifuge from human serum shows two major components termed as HDL_2 (d. 1.063-1.125) and HDL_3 (1.125-1.210).

The lipoprotein fraction of more density than 1.210 is named VHDL, which migrates with $alpha_1$-albumin fraction in starch electrophoresis[3].

Both HDL_2 and HDL_3 are low in patients with atherosclerosis and in most of the patients the decrease in HDL_2 exceeds in that in HDL_3. Premenopausal women, having higher HDL_2 levels than men of the same age[4], may be better protected against atherosclerosis.

Perturbation of HDL subfraction concentrations can be produced by various therapeutic measures. Nicotinic acid and clofibrate therapy induces an increase in the ratio of HDL_2/HDL_3[5,6], while estrogen causes a selective rise of HDL_3 level without influencing HDL_2[7].

A d.$>$1.210 fraction (VHDL) may belong to an albumin family ($alpha_1$-albumin) and also contains phospholipids.

Because lysolecithin after LCAT reaction is transported in large part in plasma by albumin[8], lipoprotein of this density class may contribute indirectly to removal of peripheral cholesterol by HDL.

Recent studies on HDL show that phospholipids in HDL fraction brings about a selective change without influencing cholesterol concentration after vigorous physical exercise[9].

Enlightenment on HDL subfractions including VHDL and their chemical compositions would, therefore, be helpful for further clinical studies on diagnosis and therapy of atherosclerosis.

However, the complete and quantitative separation of HDL between HDL_2 and HDL_3 cannot be achieved by the conventional lipoprotein-floating method with the aid of difference in density by means of preparatory ultracentrifugation. The analytical and rate zonal ultracentrifugal techniques require acquisition of expensive equipments[10].

Determination of cholesterol and phospholipids in HDL_2, HDL_3 and VHDL subfractions in the device performed in the present study was designed by preparatory centrifuge with an application of difference in cholesterol/phospholipid ratio between HDL_2 and HDL_3 after precipitation and elimination of LDL and VLDL by polyanions.

The method demonstrated reliability with relative simplicity of the procedure for accurate quantification of serum lipoprotein lipids of high density classes.

MATERIALS AND METHODS

Subjects: Fifteen healthy subjects and 23 patients were used, the latter comprising 9 with cerebral thromobosis, 4 with myocardial infarction 10 with diabetes mellitus.

The blood samples were obtained at least 3 months after attacks of thrombosis or myocardial infarction. All the patients with cerebral thrombosis were examined by computed tomography before establishment of final diagnosis.

Blood was withdrawn from the antecubital vein early in the morning before breakfast after overnight fast.

Methods: The methods were fundamentally based on the combination of Burnstein[11] and Havel's[3] procedures. Five milliliters of serum was added with 0.5 ml of 4% sodium phosphotangstate (NaPhT) and 0.125 ml of 2 M $MgCl_2$ (density of the solution containing two substances was 1.050), which were all fully mixed. After having kept the mixture upright for 10 minutes, serum was centrifuged at 3,000 r.p.m. for 15 minutes.

After determining cholesterol and phospholipids in the supernatant of samples (non-protein density was 1.011), the supernatant fraction was added with the stock solution (d. 1.346) according to the Havel's formula (the stock solution containing 153.0 g of NaCl and 354.0 g of KBr per liter to regulate the non-protein solvent density to 1.125. This solution was then centrifuged with the angle rotor #494 of the ICE/B-60 Ultracentrifuge (Kubota-International) at 105,000xG for 22 hours. The cen-

trifuged solution was divided into four fractions, A1 (0.5 ml), A2 (1.0 ml), B (2.0 ml) and C (the remaining solution, approximately 3.5 ml). The solvent density of fraction C was then raised to 1.210 by further addition of the stock solution, and ultracentrifugation and separation were repeated (0.5 ml C1).

Cholesterol and phospholipids in each fraction were determined (A1, A2, B, C and C1).

As shown in Figure 1, separation between HDL_2 and HDL_3 or between HDL_3 and VHDL was incomplete by the centrifugation under the above mentioned condition, which may be ascribed to the following:

1. Fractions A1 and C1 contained HDL_2 and HDL_3 respectively.
2. Fraction A2 contained both HDL_2 and HDL_3 but not VHDL.
3. Fraction C contained both HDL_3 and VHDL.

Because the phospholipids/cholesterol ratio differs among these lipoprotein classes, being usually higher in HDL_3 than in HDL_2, the HDL_2 and HDL_3 ratio in fraction A2 was calculated from the phospholipids/cholesterol ratio.

$$CA1 \cdot x + CH \cdot y = CA2(x+y) \qquad (1)$$

$$PA1 \cdot x + PH \cdot y = PA2(x+y) \qquad (2)$$

$$x + y = 1 \qquad (3)$$

wherein CA1, CA2 and CH represent cholesterol (mg) in fraction A1, A2 and (B+C) respectively; PA1 and PA2, phospholipids in fractions A1 and A2 (mg); PH, phospholipids which belongs to HDL_3 in fraction B+C (this fraction contains both HDL_3-phospholipids and VHDL-phospholipids) and x : y, the volumetric ratio of HDL_2 and HDL_3 in fraction A2.

When $\frac{PA1}{CA1} = a1$, $\frac{PA2}{CA2} = a2$, $\frac{PH}{CH} = cH$,

then

$$a2 = \frac{PA2}{CA2} = \frac{PA1 \cdot x + PH \cdot y}{CA1 \cdot x + CH \cdot y} \qquad (4)$$

Substitute equations $PA1 = a1 \cdot CA1$ and $PH = cH \cdot CH$ into equation (4), then

$$\frac{y}{x} = \frac{(a1 - a2)AC1}{(a2 - cH)CH} \qquad (5)$$

PH, which is HDL_3-phospholipids in fractions B and C, was calculated by the following equation, because phospholipids in fractions B and C are not only from HDL_3 but also from VHDL.

$$PH(mg) = CH \cdot \frac{PC1}{CC1} \qquad (6)$$

wherein CC1 is cholesterol in fraction C1 and PC1, phospholipids in fraction C1. CH is assumed to be solely from HDL_3 and VHDL to contain no cholesterol, although a very small amount of cholesterol has been detected in this fraction.

Thus HDL_2-cholesterol (or phospholipids) were derived from Al-cholesterol (or phospholipids) added to a part of A2-cholesterol (or phospholipids) which was calculated by the ratio of HDL_2 to HDL_3 in fraction A2, that is $\frac{x}{y}$.

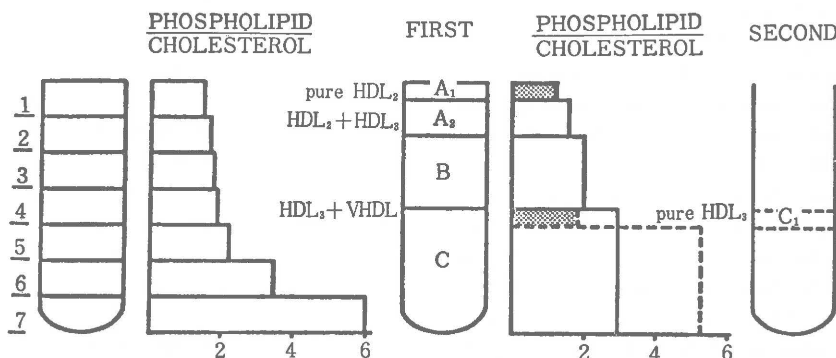

Fig. 1. Change in phospholipids/cholesterol ratio in the test tube separated into seven fractions (extreme left) after ultracentrifugation (105,000 xG, 22 hours). The ratio gradually grew larger from the surface to the bottom of the tube (block diagram on the left). Change in phospholipids/cholesterol ratio of fraction A1-C (test tube in the middle) after first centrifugation (solid line) and of fraction C (test tube on extreme right) after second centrifugation (broken line) (block diagram on the right).

HDL_3-cholesterol and HDL_3-phospholipids were also derived likewise.

VHDL-phospholipids were calculated by the following.

VHDL-phospholipids = phospholipids in fractions B and C − PH ……………………(7)

VHDL-phospholipids were also obtained by another formula in some cases.

(Phospholipids in supernatant after precipitation by polyanions) − (HDL_2-phospholipids + HDL_3-phospholipids) ……………………(8)

Cholesterol in each fraction was determined by enzymatic method reported by Allain et al.[12] Phospholipids were determined by enzyme method with an application of choline determination described by Takayama et al.[13]

RESULTS

1. Reproducibility of lipid concentrations in fractions of test tube and reproducibility of lipids in HDL_2 and HDL_3:

Forty milliliters of serum (HDL-cholesterol, 50 mg/dl) was served for analysis of reproducibility in the present method. The coefficient of variation (C. V.) was 1.62–2.69% in fractions B and C which had a large volume. On the other hand, C. V. was 4.58–6.18% in fractions A1, A2 and C1 which had a small volume.

In contrast to the above results that C. V. was variable according to the volume. the phospholipids/cholesterol ratio was nearly constant and C. V. s of fractions A1, A2 and C1 were 0.91, 0.89 and 1.57% respectively. C. V. s of cholesterol and phospholipid concentrations were approximately 10% in HDL_2, approximately 5% in HDL_3 and 8.7% in VHDL.

2. VHDL-phospholipids as calculated by two procedures and possibility of contamination of VHDL in fraction B:

The columns (A) and (B) are VHDL-phospholipids calculated by two procedures. Phospholipids in fraction A2 does not participate in equation (A) but participates in equation (B). Therefore, if fraction (B) was contaminated with a large amount of VHDL-phospholipids, the figures in column (B) would always be larger than those in column (A), and (C) would be the amount of phospholipids in fraction B when experimental errors were negligible. But the averages from 10 samples calculated by these two procedures were 16.5±9.3 mg/dl and 16.3±11.3 mg/dl, with negative figures in column (C) in half the samples. These results support that there was not or was merely a trace amount of contamination of VHDL-phospholipids in fraction B.

Table 1. Reproducibility of cholesterol and phospholipids in fractions A1–C

		Ch	PL	PL/Ch
A1	m	9.41	14.51	1.541
	s. d.	0.43	0.68	0.014
	C. V.	4.58	4.65	0.910
A2	m	9.99	15.79	1.580
	s. d.	0.53	0.78	0.014
	C. V.	5.29	4.95	0.890
B	m	14.98	25.58	1.710
	s. d.	0.24	0.49	0.025
	C. V.	1.62	1.91	1.460
C	m	13.34	40.64	3.060
	s. d.	0.25	1.10	0.102
	C. V.	1.87	2.69	3.330
C1	m	2.39	3.85	1.678
	s. d.	0.12	0.24	0.026
	C. V.	5.02	6.18	1.530

m mean (mg/dl)
s. d. standard deviation (mg/dl)
C. V. coefficient of variation (%)
Ch Cholesterol
PL Phospholipids

Table 2. Reproducibility of cholesterol and phospholipids in HDL-subfractions

	HDL2		HDL3		VHDL-
	Ch	PL	Ch	PL	PL
m	14.80	22.90	32.90	53.40	20.20
s. d.	1.47	2.34	1.58	2.63	1.76
C. V.	9.93	10.21	4.80	4.93	8.72

m mean (mg/dl)
s. d. standard deviation (mg/dl)
C. V. coefficient of variation (%)
Ch Cholesterol
PL Phospholipids

3. Cholesterol and phospholipids in HDL-subfractions in healthy subjects:

The individual and average cholesterol and phospholipid concentrations in HDL_2, HDL_3 and VHDL are listed in Table 4. The average cholesterol and phospholipids in these fractions were lower in men than in women.

4. Cholesterol and phospholipids in HDL-subfractions in cerebral thrombosis, myocardial infarction and diabetes mellitus:

Cholesterol and phospholipids in HDL_2 and HDL_3 were low in patients with cerebral throm-

Table 3. VHDL-phospholipids calculated by two procedures

	(A)	(B)	(C)
1	17.5	16.3	−1.2
2	19.2	14.1	−5.1
3	13.4	14.7	+1.3
4	40.3	46.8	+6.5
5	18.1	16.8	−1.3
6	17.9	16.6	−1.3
7	10.7	8.4	+2.3
8	12.5	14.9	+2.4
9	6.7	7.2	+0.5
10	9.1	7.6	−1.5
	16.5±9.3	16.3±11.3	

(A) = Whole phospholipids in fracions B and C minus HDL_3 phospholipids in fracions B and C (mg/dl)
(B) = Whole phospholipids after precipitation by polyanions minus(HDL_2 phospholipids + HDL_3 phospholipids) (mg/dl)
(C) = (B) − (A)

bosis and myocardial infarction. In diabetic patients these lipids decreased as compared with the normal subjects. But the decrease of lipids in HDL_2 was less in disbetic patients when compared with patients with cerebral thrombosis and myocardial infarction, resulting in the higher average of HDL-cholesterol and HDL-phospholipids in the former disease than in the latter two.

While VHDL-phospholipids decreased significantly in patients with cerebral thrombosis in most cases, they did not decrease in patients with myocardial infarction except for the case, HY and in diabetic patients except for two cases, RI and KI.

Table 4. Cholesterol and phospholipids in HDL_2 and HDL_3 and phospholipids in VHDL in healthy subjects

	HDL		HDL_2		HDL_3		VHDL
	Ch	PL	Ch	PL	Ch	PL	PL
(Male)							
Y.M.65	49.7	108.7	12.4	21.0	37.3	69.7	17.5
N.M.66	51.4	103.1	17.3	27.5	34.1	56.4	19.2
S.Y.56	48.7	75.7	12.7	15.2	36.0	48.7	11.8
K.I.69	66.5	108.4	22.3	28.4	46.2	65.1	14.9
R.S.71	56.4	102.4	12.9	17.6	43.5	63.3	21.5
L.D.65	53.7	114.8	8.1	21.8	46.6	69.1	23.8
M.D.52	51.4	98.5	17.3	29.0	34.0	42.5	26.9
K.I.62	50.0	103.5	15.8	30.9	34.1	44.2	28.1
m	53.4	101.8	14.8	23.9	37.6	57.3	20.4
s. d.	5.8	11.6	4.2	5.8	7.8	11.0	5.7
(Female)							
Y.U.55	58.5	116.3	18.6	28.3	39.9	66.5	21.5
A.I.47	72.6	148.6	19.2	30.7	53.4	93.5	24.4
S.M.56	68.7	119.7	27.8	39.6	40.9	62.1	18.0
U.N.62	70.4	131.3	11.8	18.3	58.6	95.7	17.3
K.T.77	43.2	97.6	15.4	23.0	27.8	43.9	30.7
T.H.72	46.1	87.3	17.3	27.5	28.7	42.6	17.6
N.K.54	54.0	103.6	21.8	32.7	33.0	50.0	20.9
m	59.1	117.8	18.8	28.5	40.3	64.9	21.4
s. d.	11.9	21.3	5.0	6.8	11.9	22.1	4.8

Ch Cholestesterol
PL Phospholipids
m mean
s. d. standard deviation

5. Fractional phospholipids in VHDL in

Table 5. (1) Cholesterol and phospholipids in HDL subfractions in patients with cerebral thrombosis

	Age	HDL		HDL_2		HDL_3		VHDL
		Ch	PL	Ch	PL	Ch	PL	PL
F. Y.	74F	36.5	69.3	4.2	5.6	32.3	51.4	12.3
T. K.	85F	41.9	82.1	11.3	18.9	30.6	55.7	7.5
A. I.	73F	28.1	54.3	6.1	9.2	22.0	34.4	10.7
N. H.	64F	29.6	79.6	6.3	10.9	23.1	43.8	24.8
H. M.	60F	27.6	43.2	3.7	7.5	23.8	36.8	8.8
M. H.	77M	30.2	60.5	5.2	7.1	25.0	39.6	13.8
J. I.	72M	32.0	52.1	7.3	9.2	24.7	38.9	4.0
M. T.	68M	32.9	68.4	7.4	12.8	25.4	42.5	13.2
T. C.	70M	32.7	66.9	6.6	11.2	26.1	50.3	5.3
	m	32.3***	64.0***	6.4***	10.2***	25.8***	43.7**	11.1***
	s. d.	4.4	12.7	2.2	3.9	3.4	7.2	6.1

Table 5. (2) Cholesterol and phospholipids in HDL subfractions in patients with myocardial infarction

	Age	HDL		HDL_2		HDL_3		VHDL
		Ch	PL	Ch	PL	Ch	PL	PL
H. Y.	62F	35.5	78.2	4.9	23.3	30.6	48.2	6.7
K. H.	77M	33.5	83.3	9.6	17.2	23.9	44.7	21.4
Y. H.	38M	40.8	82.4	7.6	10.7	33.2	52.8	18.9
N. U.	67M	28.5	63.6	3.3	4.0	25.2	37.9	21.7
	m	37.1**	76.9**	6.3**	13.8**	28.2*	45.9*	17.2N.S.
	s. d.	7.6	9.1	2.8	8.3	4.4	6.3	7.1

(3) Cholesterol and phospholipids in HDL subfractions in patients with diabetes mellitus

	Age	HDL		HDL_2		HDL_3		VHDL
		Ch	PL	Ch	PL	Ch	PL	PL
R. I.	76F	24.9	51.7	7.4	10.2	17.5	28.1	13.4
H. S.	71F	44.9	98.0	19.0	32.0	25.9	46.3	19.2
T. M.	68F	56.1	120.5	16.2	28.7	39.9	51.5	40.3
M. H.	66F	34.3	81.3	8.2	14.1	26.1	49.1	18.1
M. K.	76F	37.3	92.5	10.0	17.6	27.3	49.8	25.5
K. K.	23F	47.4	101.7	13.7	19.6	33.7	51.6	30.5
S. K.	55F	39.3	101.2	11.7	20.8	27.6	50.7	29.7
K. Y.	39M	53.6	97.8	17.5	25.6	36.1	46.9	25.4
S. T.	48M	25.8	74.2	12.1	17.0	13.7	38.1	18.1
K. I.	72M	51.7	95.6	22.8	33.8	28.9	49.3	12.5
	m	41.5**	91.5*	13.9N.S.	21.9N.S.	27.7**	46.1*	23.3N.S.
	s. d.	11.1	18.6	5.0	7.8	7.9	7.5	8.7

Ch: Cholesterol, PL: Phospholipids, F: Female, M: Male
***$p<0.001$ **$p<0.01$ *$p<0.05$ Statistically significant decrease as compared with normal healthy subjects

Table 6. Fractional phospholipids of VHDL in health and cerebral thrombosis

	Cephalin & others	Lecitin	Sphyngo-myelin	Lyso-lecithin
Normal	1.28	2.04	1.64	15.45
	0.69	2.17	1.49	13.15
	1.03	1.90	1.52	17.04
	1.17	2.52	2.02	18.68
m	1.04	2.15	1.66	16.08
s. d.	0.25	0.26	0.24	2.35
Cerebral	0.35	0.77	0.42	2.46
Thrombosis	0.69	1.35	1.16	9.10
	1.14	1.74	1.77	9.15
	0.54	1.43	1.56	7.17
m	0.68	1.32	1.22	6.97
s. d.	0.33	0.40	0.59	3.14
	N.S.	$p<0.05$	N.S.	$p<0.01$

healthy subjects and patients with cerebral thrombosis:

Table 6 lists four main fractional phospholipids in VHDL separated by the thin layer chromatography in four healthy subjects and four patients with cerebral thrombosis.

Lecithin and lysolecithin were significantly lower in these patients than in healthy subjects. Because lysolecithin was the majority in VHDL-phospholipids, the decreased VHDL-phospholipids seen in these patients seemed attributable to the decrease in lysolecithin in the VHDL fraction.

DISCUSSION

Havel et al.[3] separated lipoprotein density classes from 1.006 to 1.063 slicing the clear zone in the middle beneath the layer at the top. But as seen in Figure 1, the clear zone of high density classes contain a good deal of cholesterol and phospholipids, which is an

indication of inadequate isolation of HDL_2 from HDL_3 at 10, 500xG for 22 hours.

They performed ultracentrifugation with the J rotor of the Spinco Model E ultracentrifuge at 173, 000xG for 18 hours and reported that 40 rotar driven for 48 hours produced the same results[8].

These methods, however, are time-consuming or require a unusual rotor.

Reproducibility of lipid determination of HDL_2 and HDL_3 by the present method is simpler and satisfies the clinical purpose.

Precise collection of each fraction after ultracentrifugation is important for reproducibility.

The tube-slicing device reported by Randolph and Ryan[14] is helpful for precise reproducibility but again is time-consuming. In the present experiment of the authors, a plastic syringe was used which was fixed to a heavy iron stand and equipped with a 18G×1½″ injection needle, the end which was cut horizontally to obtain toughness on the surface of the liquid.

A volume of 0.5 ml for fractions A1 and C1 is the maximum volume for acceptable accuracy. Fraction A2 (1.0 ml) has to be a mixture of HDL_2 and HDL_3 contaminated with the least possible amount of VHDL. No contamination of VHDL in fraction A2 was predicted, which was endorsed by the fact that the average values of VHDL-phospholipids as calulated by two procedures proved almost identical with each other as shown in Table 3.

Concentration of cholesterol and phospholipids in HDL_2 was less than those in HDL_3 and concentration of phospholipids changed in parallel with that of cholesterol. VHDL-phospholipids was the least of all other lipoprotein phospholipids among three lipoprotein classes. In all three lipoprotein classes there were more concentrations of these lipids in female than in male.

Both lipids of HDL_2 and HDL_3 significantly dereased in patients with cerebral thrombosis and myocardial infarction. In well controlled diabetes mellitus, the concentration of lipids in HDL subfraction was shown to vary from the normal to the very low value. On an average, however, the decrease of lipids in HDL_2 was less in these patients as compared with patients with cerebral thrombosis and myocardial infarction.

Havel et al. reported that VHDL-phospholipids (d.>1.210) was soluble in ethanol-acetone, that six young adults had 15 to 27 mg of 1.210 density infranate per 100 ml of serum phospholipids and that there was no difference in its concentration between them and eight hyperlipidemic individuals[3].

The results of the present study showed the significant decrease in VHDL-phospholipids in patients with cerebral thrombosis.

In the previous report of the authors, phospholipids in liporoteins of density below 1.063 (α-lipoprotein phospholipids) had been determined by precipitation of VLDL and LDL with polyanions (Na phosphotangstate and $MgCl_2$) to find that phospholipids in this fraction decreased in atherosclerotic patients, particularly in cerebral thrombosis[15].

The present study clarified that decreased phospholipids in this fraction is due not only to the decrease in phospholipids in HDL_2 and HDL_3 but also in VHDL-phospholipids.

The clinical significance of lowered VHDL-phospholipids and its relationship to the pathogenesis of atherosclerosis are not known at the present time as yet.

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