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COMPARATIVE DETERMINATION OF PLASMA PHOSPHOLIPIDS BY AUTOMATED GAS—LIQUID CHROMATOGRAPHIC AND MANUAL COLORIMETRIC PHOSPHORUS METHODS

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SUMMARY

Plasma samples obtained during a prevalence study of hyperlipemia in a free-living urban population were analyzed for phosphatidylcholine, sphingomyelin and lysophosphatidylcholine content by automated high-temperature gas—liquid chromatographic (GLC) and manual colorimetric phosphorus (thin-layer chromatographic, TLC) methods. The GLC estimates were obtained from a quantitative analysis of the diacylglycerol, ceramide and monoacylglycerol moieties released from the parent phospholipids by digestion with phospholipase C, while the TLC estimates were derived by manual colorimetric phosphorus analyses of the individual phospholipid classes resolved by TLC. On samples analyzed over a two-year period the methods gave excellent correlation for the total phospholipids ($r = 0.98$), phosphatidylcholine ($r = 0.98$) and sphingomyelin ($r = 0.90$), but resulted in a poor agreement for lysophosphatidylcholine ($r = 0.69$). Comparable results were obtained for estimates of these phospholipids in plasma very low density, low density and high density lipoproteins. The between-method coefficient of variation ranged from 3 to 5% for phosphatidylcholine and from 5 to 10% for sphingomyelin. The relative error for the estimates of lysophosphatidylcholine ranged from 10 to 25%, and was due to the inclusion in the GLC estimates of a variable proportion of plasma free monoacylglycerols. Other differences between the two methods are due to various analytical errors and biases inherent in the two techniques. The within-day, within GLC, relative error averaged 1% for phosphatidylcholine, 3% for sphingomyelin and 5% for lysophosphatidylcholine. The apparent high precision and accuracy of the GLC method recommend it as an alternative to conventional direct methods of phospholipid analyses based on TLC isolation of lipid classes and colorimetric measurements of their phosphorus content. The GLC analyses of the plasma phospholipids are particularly convenient in conjunction with GLC measurements of plasma cholesterol and triacylglycerols, where a smaller throughput of samples is not a limitation and where both total amount and relative proportion of the lipids are of interest.

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INTRODUCTION

Individual neutral lipid classes of plasma can be readily separated and quantitatively estimated by conventional [1—5] and automated [6—11] high-temperature gas—liquid chromatography (GLC). The results obtained for plasma total cholesterol and total triacylglycerols compare closely to those derived by automated chemical methods (AutoAnalyzer) of analysis, when calibration is made with plasma or serum [6, 9]. These estimates may also include the neutral lipid moieties present in the phospholipids, provided they are quantitatively released by dephosphorylation with phospholipase C [2, 4, 10] or by pyrolysis [1], but extensive comparisons with chemical methods of analysis have not been made.

In the following study we have compared the results of the automated GLC and the manual phosphorus methods of analysis of phospholipids in over 100 samples of plasma and of individual lipoprotein fractions from normal subjects and patients with hyperlipoproteinemia. In general, the GLC method gives values which are within 5—10% of those obtained by the chemical assay. The differences are traced to various inherent errors and biases in the two methods of analysis.

MATERIALS AND METHODS

The synthetic neutral lipid and free fatty acid mixtures along with the tridecanoylglycerol internal standard were available in the laboratory from previous studies [9]. The synthetic samples of glycerophospholipids and sphingomyelins were purchased from Applied Science Labs., State College, Pa., U.S.A. The plasma samples of normal subjects and patients with hyperlipoproteinemia as well as the lipoprotein fractions were supplied by the Toronto-McMaster Lipid Research Clinic, Toronto, Canada. The lipoprotein fractions [very low density (VLDL), $d < 1.006$; low density (LDL), $d = 1.006—1.063$; high density (HDL_2), $d = 1.063—1.125$; HDL_3, $d = 1.125—1.21$] had been obtained by means of ultracentrifugation according to the description of Hatch and Lees [12]. Phospholipase C (α-toxin of *Clostridium welchii*, Type I) was purchased from Sigma, St. Louis, Mo., U.S.A. The TRISIL/BSA was obtained from Pierce, Rockford, Ill., U.S.A. Other reagents and solvents were of Fisher certified reagent grade and were not further purified. All glass-ware was rinsed with chloroform—methanol (2:1) prior to use.

Preparation of total lipid extracts

Total lipid extracts of plasma and of plasma lipoproteins were obtained with chloroform—methanol (2:1) essentially, as described by Folch et al. [13]. Plasma samples (0.2 ml each) were pipetted directly into 15-ml centrifuge tubes containing 3 ml of ice-cold methanol. After mixing on a Vortex mixer, 6 ml of chloroform were added to each tube and the contents were again mixed. The tubes were then left at room temperature for 30 min with occasional stirring. After adding 2.0 ml of 0.9% NaCl, the tubes were subjected to rigorous mixing and were centrifuged. The lower phases were withdrawn quantitatively to 8-ml glass vials after passing through a Pasteur pipette column of anhydrous Na_2SO_4 and were evaporated in a stream of nitrogen. To the centrifuge tube containing

residual plasma proteins and upper phase, 6 ml each of Folch lower phase were added. The tubes were stirred thoroughly for 3 min and centrifuged. The lower phases were collected into the original vials and dried. The process was repeated one more time. The extracts were not backwashed. To check the efficiency of the extraction method, the plasma samples were extracted three times with 9 ml each of chloroform—methanol—HCl (200:100:0.1). After centrifugation, the pooled extracts were washed with 1 *N* HCl, the lower phases neutralized with ammonia and washed with Folch upper phase containing NaCl. The lower phases were dried and subjected to phosphate analyses and/or thin-layer chromatography (TLC). The extent of extraction by the two methods was further ascertained by saponification of the residual protein from both extraction procedures with 1 *N* KOH, extracting the acidified reaction mixture with diethyl ether and quantitating any fatty acids recovered by GLC [14].

Preparation of neutral lipid extracts

Total neutral lipids of plasma were obtained by treatment of an isopropanol extract of plasma (19:1) with Zeolite [12], which removed phospholipids and other polar components but retained free fatty acids along with the neutral lipids in the solution. Alternatively, neutral lipids were isolated from the solvent front of the TLC plates developed with phospholipid solvents from total lipid extracts of whole plasma or of individual lipoprotein classes (see below).

Hydrolysis with phospholipase C

The plasma samples were digested with phospholipase C as previously described [2, 9]. EDTA (0.01%) plasma (0.2—0.5 ml) was added to a solution of 0.2—0.4 mg (1—2 units) of phospholipase C in 2—3 ml of 17.5 m*M* Tris buffer (pH 7.3) and 1.3 ml of 1% $CaCl_2$, and 1 ml of diethyl ether. The mixture was incubated with stirring for 2 h in tightly closed screw-cap vials at 30°. The reaction was terminated by the addition of five drops of 0.1 *N* HCl and extracting once with 10 ml of chloroform—methanol (2:1) containing 150—250 μg of tridecanoylglycerol. The solvent phases were separated by centrifuging for 10 min at 200 *g* after each extraction. The clear chloroform phase was passed through 2 g of anhydrous Na_2SO_4. The effluent was evaporated to dryness and diluted to a known volume from which aliquots were taken for the determination of the total lipid profile by GLC, and of any residual phospholipids by TLC and phosphorus determination.

Plasma lipoproteins were digested with phospholipase C under the general conditions described for whole plasma except that the Ca^{2+} concentration in the digestion medium was increased ten-fold to overcome the chelating effect of the extra EDTA present in the sedimentation media [9].

Thin-layer chromatography

For the measurement of phospholipid class composition the total lipid extracts were chromatographed on thin layers of silica gel H as described by Shaikh and Palmer [15]. The lipid extracts were applied as 2 cm wide bands to TLC plates preactivated for 1 h at 120°. The chromatograms were developed in paper-lined tanks containing chloroform—methanol—acetic acid—water (100:

45:20:7) as the developing solvent. The phospholipids were identified by comparison with known standards and were located after exposure to iodine vapour and/or spraying the plates with the acid molybdate reagent of Dittmer and Lester [16].

Total neutral lipids were recovered as a single band from the top of the 2',7'-dichlorofluorescein-sprayed TLC plates and, after elution with chloroform, were silylated and subjected to GLC examination as described below.

Analysis of phosphorus

The phospholipid contents of any thin-layer fractions were determined by the method of Bartlett [17] after direct digestion of the lipid-containing silica gel in perchloric acid—sulfuric acid (1:2) mixture. The phospholipid content of total lipid extracts was determined directly. Standard curves for phosphate were prepared daily using appropriate reagents. The error of the method was ± 1% for components making up more than 5% of the total.

Automated gas—liquid chromatography

The automated analyses of the plasma and lipoprotein total lipids were performed following preliminary dephosphorylation and trimethylsilylation as previously described [7, 9]. For this purpose a Hewlett-Packard Model 5700 automatic gas chromatograph was equipped with dual nickel columns (54 × 0.2 cm I.D.) packed with 3% OV-1 on Gas-Chrom Q (100—120 mesh) as supplied by Applied Science Labs. The peak areas were measured by means of an electronic integrator, the output of which was recorded on a punched paper tape. The tape was processed by means of a Hewlett-Packard 8900 calculator using appropriate computer programs as previously described [6, 7]. The peak areas were calculated in relation to the tridecanoylglycerol internal standard and were expressed as mg%.

Calculations

Total phospholipid content of a sample was determined by summing the areas of peaks 22—24 and 34—42, and multiplying by calibration factors derived from standard lysophosphatidylcholine (LPC), phosphatidylcholine (PC) and sphingomyelin (SPH). Due to frequent poor reproducibility of the automated integration of the peak areas in the C_{40}—C_{42} carbon number region, an alternative calculation was devised to obtain the total PC content from the areas of carbon numbers C_{36} and C_{38}, and of SPH from the area of carbon number C_{34}, all of which are easily measured, as follows. Total PC = total DG × 1.28, where total DG = corrected $(C_{36} + C_{38})/0.81$, and corrected $C_{36} + C_{38} = (C_{36} + C_{38}) - C_{34}$ ceramide; with C_{34} ceramide = $C_{34} - 0.051 \times C_{36}$. The total SPH = total ceramide × 1.28, where total ceramide = C_{34} ceramide × (0.758/0.30). The factor 0.758 is the ratio of the response factors for ceramide and diacylglycerol trimethylsilyl (TMS) ethers (0.758=0.681/0.898). The factors 0.81 and 0.30 represent the diacylglycerol and ceramide fractions, respectively, measured in the total PC and SPH from large plasma pools. The multiplication factor 1.28 converts the ceramide and diacylglycerol moieties into the corresponding phosphorylcholine derivatives.

Statistical analyses

The evaluation of the GLC procedure for phosphatidylcholine and sphingomyelin determination was modeled on a comparable study of methodology reported for total cholesterol and triacylglycerols [9]. Systematic errors were measured by the difference between average GLC values and the manual phosphorus or target values using linear regression and correlation methods [18]. Random error was estimated by the variance or standard deviation. The relative error values are averages of percentage deviations defined as: relative error = (GLC value − phosphorus value) × (100/phosphorus value). A coefficient of variation between duplicates was calculated using the formula C.V. % = $(100\sqrt{d^2/2})/\bar{X}$, where d is the difference between duplicates and $\bar{X}$ is the mean.

The within-day standard deviation was used as the measure of within-day variability. The overall standard deviation was used as the measure of the variability of a single determination of a quality control sample by the GLC method.

RESULTS

Molecular weight distribution of plasma phosphatidylcholines and sphingomyelins

Fig. 1 shows the carbon number profiles of the diacylglycerol moieties of the total plasma phosphatidylcholines and of the ceramides of total plasma sphingomyelins following isolation of each phospholipid class by TLC, enzymic dephosphorylation and trimethylsilylation. It is seen that the diacylglycerol moieties range in carbon number from 34 to 42, with the carbon numbers 34 and 42

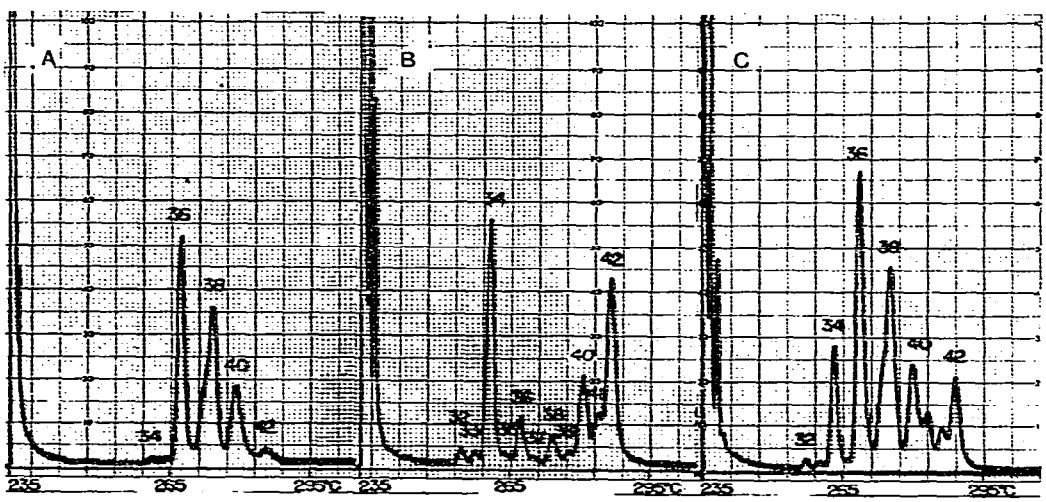

Fig. 1. GLC analyses of diacylglycerol and ceramide moieties of choline phospholipids of whole plasma following a prior TLC isolation, enzymic dephosphorylation and trimethylsilylation. A, Diacylglycerols; B, ceramides; C, mixture of 60 parts A and 40 parts B. Peaks 34—42, TMS ethers of diacylglycerols with 32—40 acyl carbons; peaks 32—42, TMS ethers of ceramides with 30—40 ceramide carbons. Temperature program as shown. Other GLC conditions are given in the text.

contributing only minor amounts (<5%). The ceramide moieties of the plasma sphingomyelins also range in carbon number from 34 to 42, but in this case the carbon numbers 34 and 42 make major contributions to the total (<30%). Dietary fats have a marked influence on the proportion of carbon numbers 36 and 38 of the diacylglycerol moieties of plasma phosphatidylcholines, but no significant changes take place in the overall carbon number range on normal diets. In general these lipid profiles hold also for the individual lipoprotein classes, but there are certain differences that require analytical attention. Both dietary and lipoprotein differences are illustrated in Table I. The samples were

TABLE I

CARBON NUMBER DISTRIBUTION OF PHOSPHATIDYLCHOLINES FROM PLASMA LIPOPROTEINS OF NORMOLIPEMIC SUBJECTS ON SATURATED AND UNSATURATED FAT DIETS

The isocaloric diets contained 20% protein, 40% carbohydrate and 40% fat. The fats had polyunsaturated:saturated fatty acid ratios of 0.25 (saturated fat) and 4 (polyunsaturated fat) [A. Kuksis et al. (1975), unpublished results]. Values are given as the mean ± S.D. for four subjects.

Carbon No.	Saturated fat			Unsaturated fat		
	VLDL (%)	LDL (%)	HDL_3 (%)	VLDL (%)	LDL (%)	HDL_3 (%)
34	1.9±0.5	3.1±0.3	2.5±0.2	1.9±0.4	3.7±0.3	2.1±0.4
36	42.2±2.6	41.1±0.9	36.4±1.2	38.7±4.1	40.4±3.9	34.6±3.6
38	38.7±2.7	37.9±1.6	40.1±0.8	43.7±2.2	40.4±2.4	42.8±1.9
40	15.1±1.0	15.4±0.4	18.3±0.6	13.5±2.9	12.6±1.4	17.2±2.8
42	2.1±0.5	2.5±0.3	2.7±0.5	2.2±0.4	2.9±0.5	3.4±0.8
36+38	80.9±2.0	79.0±1.2	76.5±1.0	82.4±	80.8±3.1	77.4±3.0

taken from a controlled dietary study lasting two weeks on each diet and involved four normolipemic individuals [Kuksis et al. (1975), unpublished results]. It is seen that the unsaturated diet caused an increase in the proportion of the diacylglycerols with carbon number 38 in the VLDL, LDL and HDL_3, while the saturated diet increased the proportion of the diacylglycerols with carbon number 36 in these lipoprotein classes. On both diets the diacylglycerols of carbon number 40 were increased in the HDL fraction over that in any other plasma lipoprotein class. Despite these variations in the carbon number proportions, the sum of the carbon numbers 36 and 38 accounted for 81±1% of total diacylglycerol species on both diets in the VLDL and LDL fractions, while in the HDL fraction, this sum accounted for 77±1% of the total on both diets. In the total plasma lipid profile the sum of carbon numbers 36 and 38 averaged 80±2% of the total diacylglycerol species and could be used as an effective basis of quantitating total plasma phosphatidylcholines when difficulties were experienced with the resolution of the higher-molecular-weight peaks or with the automatic slope sensor of the peak area integrator. Under normal conditions diacylglycerophospholipids other than phosphatidylcholines would not be expected to contribute more than a few per cent of the total glycerophospholipid of whole plasma or of any plasma lipoprotein fractions [19].

Short-term (two weeks) dietary regimens had a slight effect on the carbon number profiles of the ceramide moieties of the plasma sphingomyelins. Greater differences were seen in the ceramide profiles of the different plasma lipoproteins. Fig. 2 compares the ceramide profiles of the LDL and HDL_3 lipo-

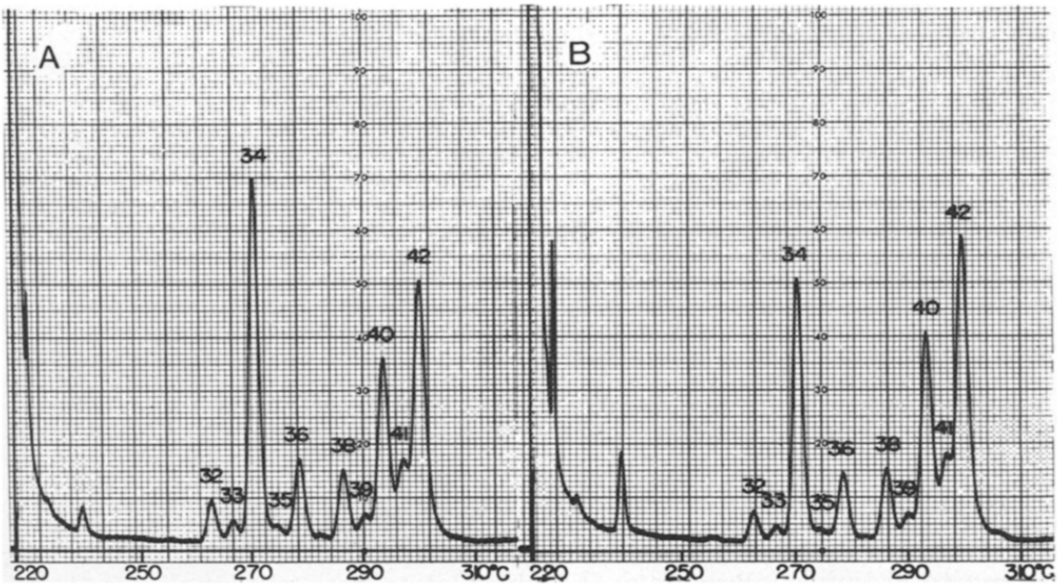

Fig. 2. GLC analyses of ceramide moieties of sphingomyelins of plasma lipoproteins of a healthy male. A, LDL; B, HDL_3. Peaks 32—42, *tert.*-butyldimethylsilyl ethers of ceramides with 30—40 ceramide carbons. Temperature program as shown. Other GLC conditions as given in the next.

proteins of a representative normolipemic subject. There is a significantly higher proportion of the longer chain ceramides in the HDL_3 than in the LDL fraction although there is no change in the overall range of the carbon numbers. Table II gives the carbon number proportions in the ceramides of the LDL and HDL_3 fractions obtained from four normolipemic subjects on free-choice diets. It is seen that the total carbon number range remains the same for all subjects in both lipoprotein classes, but that the proportion of the higher-molecular-weight species is significantly greater in the HDL_3 fraction. Carbon number 34 ceramides make up an average of 33% of the total ceramide population in the LDL and 24% in the HDL_3 fraction. These proportions can be used as a reliable basis for the calculation of the total ceramide content in these plasma lipoproteins [20]. Likewise, the total ceramide or sphingomyelin content of whole plasma could be calculated from the total lipid profile of the plasma on the basis of an average contribution of 30% for carbon number 34 to the total plasma sphingomyelin. This estimate, however, could be in serious error if the LDL and HDL proportions were significantly altered in a given plasma sample. The overall error of analysis of the isolated mixtures approaches the error of the reproducibility of the chromatographic system [6, 7, 9].

TABLE II

CARBON NUMBER DISTRIBUTION OF SPHINGOMYELINS FROM LOW AND HIGH DENSITY LIPOPROTEINS OF NORMOLIPEMIC SUBJECTS ON FREE-CHOICE DIETS

Subjects as in Table I. Kuksis et al. (1975), unpublished results. Underlined values are specifically mentioned in the text. Values are given as per cent of total.

Carbon No.	Subject 1		Subject 2		Subject 3		Subject 4		Average	
	LDL	HDL_3	LDL	HDL_3	LDL	HDL_3	LDL	HDL_3	LDL	HDL_3
32	3.1	2.7	4.3	3.5	2.7	1.8	2.5	1.7	3.2	2.4
33	1.8	1.4	2.3	1.6	1.6	1.0	1.6	0.9	1.8	1.2
34	31.4	24.3	33.1	26.0	33.7	22.7	35.0	24.3	33.3	24.3
35	1.3	0.9	1.5	1.1	1.2	0.8	2.0	0.6	1.5	0.8
36	7.5	6.5	7.3	6.8	6.6	5.7	8.6	6.9	7.5	6.5
37	0.5	0.7	0.9	0.5	0.6	0.4	0.6	0.1	0.7	0.4
38	6.0	6.4	5.4	5.4	4.9	5.5	4.6	4.2	5.2	5.4
39	2.0	1.9	2.1	2.1	1.8	1.8	1.2	0.9	1.8	1.7
40	16.1	18.9	14.6	17.7	15.7	20.2	12.4	15.3	14.7	18.0
41	5.6	5.5	6.2	6.2	5.4	5.9	4.8	6.3	5.5	6.0
42	24.4	30.3	21.9	28.9	24.7	32.7	26.5	38.6	24.4	32.6
43	0.4	0.8	0.6	0.4	0.9	1.5	0.2	0.1	0.5	0.7

Interference from free mono- and diacylglycerols and ceramides

Fig. 3 shows the GLC profiles of the plasma neutral lipids and of the corresponding total lipids following dephosphorylation with phospholipase C. It is seen that the neutral lipids of a normolipemic subject contain very little free diacylglycerol (peaks 36 and 38) or free ceramides (peak 34). Furthermore, there is very little overlap between the longer chain diacylglycerols and ceramides, and the shorter chain cholesteryl esters (peak 41). There is also very little free monoacylglycerol (peaks 22 and 24) in the neutral lipid chromatograms. A marginal increase takes place following phospholipase C hydrolysis of the plasma lysophosphatidylcholines, which, however, is not complete (see below). In addition, the monoacylglycerol peaks are split up into two or three components due to a partial resolution of the saturated and unsaturated species and of the positional isomers (*sn*-1(3)- and *sn*-2-enantiomers). The unsaturated monoacylglycerols migrate ahead of the saturated ones, as do the *sn*-2-isomers, when compared to the *sn*-1(3)-isomers, although not to the same extent. As a result the quantitation of the monoacylglycerols by GLC is erratic and accurate estimates of lysophosphatidylcholines may be difficult to obtain. Table III gives the results of quantitative estimation of free diacylglycerols and monoacylglycerols in the plasma of a representative number of normolipemic and hyperlipemic subjects selected at random from an urban population. It is seen that the free diacylglycerols (peaks 36 and 38) range from 2 to 59 mg% over a triacylglycerol range of 65—2032 mg% and are related closely to the total lipid and especially the total triacylglycerol level of the plasma, $r = 0.96$ and $r = 0.95$, respectively. The correlation with phosphatidylcholine, however, was relatively poor ($r = 0.71$). Since the elevation of free diacylglycerols accompanying elevated total triacylglycerols is usually associated with an increase in plasma phospholipids, the plasma free diacylglycerols would not be expected to

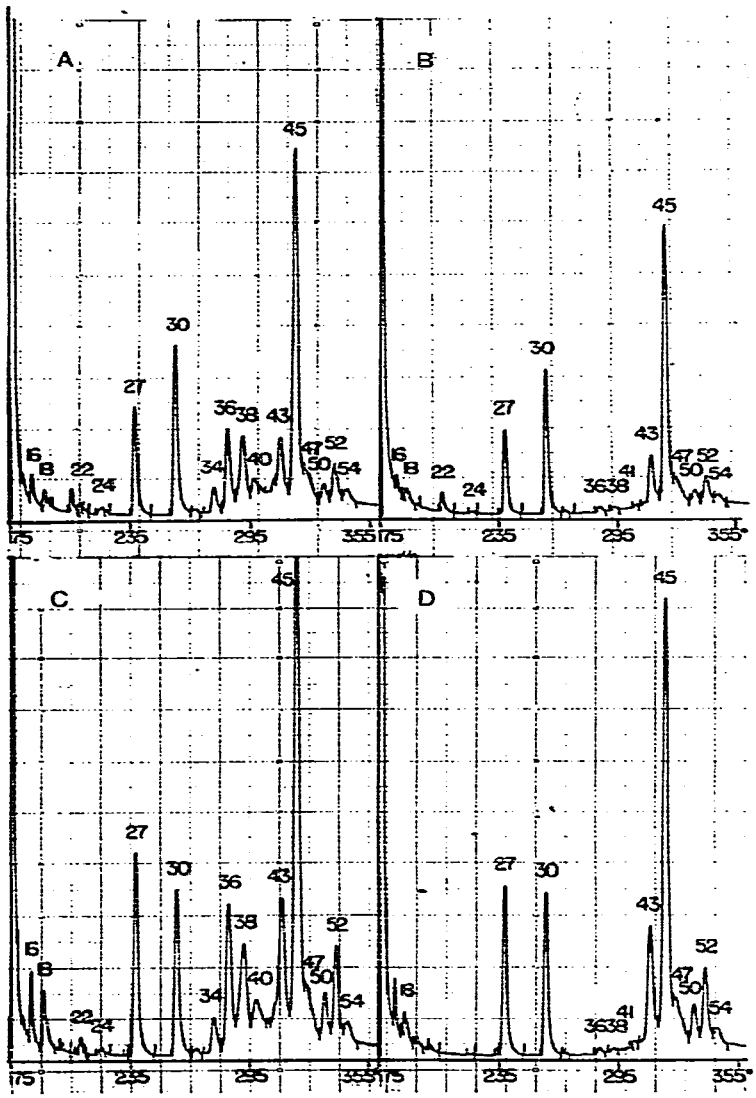

Fig. 3. GLC profiles of corresponding total and neutral plasma lipids of a normolipemic and a hyperlipemic subject. A, Total lipids of normolipemic subject; B, neutral lipids of normolipemic subject; C, total lipids of hyperlipemic subject; D, neutral lipids of hyperlipemic subject. Peaks 16 and 18, TMS esters of free fatty acids with 16 and 18 acyl carbons; peaks 22—24, di-TMS ethers of monoacylglycerols with 16 and 18 acyl carbons; peak 27, TMS ether of cholesterol; peak 30, tridecanoylglycerol (internal standard); peak 34, TMS ether of palmitoylsphingosine; peaks 36—42, TMS ethers of diacylglycerols with a total number of 34—40 acyl carbons; peaks 43—47, cholesteryl esters of fatty acids with a total number of 16—20 acyl carbons; peaks 48—54, triacylglycerols with a total number of 48—54 acyl carbons. Sample size 1 μl of an approximately 1% solution in silylation mixture. Attenuation: 100 times full sensitivity. Temperature program as shown. Other GLC conditions as given in the text.

TABLE III

ESTIMATES OF FREE MONOACYL- AND DIACYLGLYCEROL CONTENT IN PLASMA OF NORMOLIPEMIC AND HYPERLIPEMIC SUBJECTS

Plasma samples	Lipid classes* (mg%) MG	DG	TG	TC	PC	(MG/TG) x 100	(DG/TG) x 100
Normal							
1	2	4	121	202	184	1.6	3.3
2	1.9		65	170	120	2.9	
3	2	2	92	167		2	2.4
4	2	4.4	162	220	172	1.2	2.7
5	2	2	69	189	163	2.8	2.8
6	0.9	2	89	214		1.0	2.2
7	2	3.0	105	157	132	1.9	2.8
8	2.1	1.8	95	194		2.2	1.9
9	2	6.0	161	234	189	1.2	3.7
10	2	4.2	77	149	155	2.5	5.4
11	3	2.7	104	192	195	2.8	2.6
12	2	2	254	113	115	0.8	0.8
*Hyperlipemic***							
1	2	7.0	106	312	192	1.8	6.6
2	2	2.4	89	300	193	2.2	2.7
3	2	3.5	73	330	272	2.7	4.7
4	4	8.0	126	258	216	3.2	6.3
5	5	7.3	114	368	294	4.4	6.4
6	2.8	7.4	169	382	176	1.6	4.3
7	2.8	19.1	640	282	281	0.4	3.0
8	3.7	6.0	236	367	237	1.5	2.5
9	5	16.1	262	463	304	1.9	6.1
10	3	11.4	231	300	238	1.2	4.9
11	2	9.5	243	275	209	0.8	3.9
12	2	24.7	348	327	253	0.5	7.0
13	2.1	13.1	266	490	494	0.2	4.9
14	2	21.8	328	253	195	0.6	6.6
15	2	10.9	294	268	204	0.7	3.7
16	5.4	5.2	179	188	153	3.0	2.9
17	2	59.4	2008	387	517	0.01	2.9
18	6.7	9.2	423	210	184	1.6	2.2
19	9.2	42.5	851	175	209	1.1	5.0
20	13	58.1	1828	424	415	0.7	3.2
21	8	52.8	2032	365	427	0.4	2.6
22	2	9.6	194	217	166	0.1	4.9
*Intralipid****							
1	3.2	10.1	148	257	227	0.2	6.8
2	2	1.6	121	140	105	1.6	1.3
3	3	11.1	242	240	104	1.2	4.6
4	2.0	12.4	392	397	253	0.5	3.2

*MG, peaks 22 + 24; DG, peaks 34 + 36 + 38; TG, TC and PC as described in the text.

**As by phenotyping algorithm in the Manual of Laboratory Operations, Lipid Research Clinics Program, Vol.1, Lipid and Lipoprotein Analysis, NHLI, NIH, Bethesda, Md., 1974.

***Fasting samples taken 16 h after infusion of moderate doses of Intralipid.

contribute significantly (more than 5%) to the estimate for total phosphatidylcholine, as determined on the basis of the diacylglycerol released by phospholipase C.

Only the hyperlipemic samples contained a measurable peak with carbon number 34, which could have represented either free ceramide or free diacylglycerol, or both. However, even if the entire peak was attributed to ceramides, this would not alter the GLC estimate of plasma sphingomyelin significantly. These findings and their interpretation are in agreement with previous work [21], which had indicated very low levels of free ceramides in human plasma.

Table III also gives estimates for the plasma free monoacylglycerol content, which could falsely inflate the estimates for plasma lysophosphatidylcholine based on the monoacylglycerol released by phospholipase C. It is seen that the free monoacylglycerol levels range from zero to about 13 mg%, with the highest levels of monoacylglycerols again being found in the samples containing the highest total lipid and triacylglycerol levels, although the overall correlation was not as good ($r = 0.62$) as for free diacylglycerols. Very few of the hyperlipemic samples contain more than 5 mg% free monoacylglycerol. These findings also are in agreement with previous work [19]. It is shown below (see Table VI) that the total monoacylglycerol levels measured following phospholipase C digestion of plasma lysophosphatidylcholine range from 5 to 20 mg% and therefore its plasma levels are seriously compromised by the presence of free monoacylglycerols in the blood. It may also be noted that the region of the chromatogram which contains the free monoacylglycerols frequently also contains other unidentified substances at a low but variable level.

A similar examination of the total and neutral lipid profiles of the major plasma lipoprotein classes (results not shown) revealed comparable low levels of free diacylglycerols, in proportion to the triacylglycerol content of the fraction [19]. Clearly, the small amounts of free diacylglycerols and free ceramides are not likely to influence the estimation of the plasma phosphatidylcholines and sphingomyelins based on their neutral lipid moieties released by phospholipase C, either in whole plasma or in isolated fractions of plasma lipoproteins.

GLC of plasma glycerophospholipids and sphingomyelins

Table IV shows the reproducibility of the quantitative estimates for total phosphatidylcholine and sphingomyelin in whole plasma of normolipemic young adults [10] analyzed over a short period of time. A total of 17 samples was examined in quadruplicate and the results calculated separately for the carbon numbers corresponding to the major ceramides and diacylglycerols. The peak areas of C_{36} and C_{38} were estimated most precisely with an average coefficient of variation of less than 1%. The peak areas of C_{34} and C_{40} were also estimated with a relatively high precision as indicated by an average coefficient of variation of less than 2%. The peak areas for C_{41} and C_{42} were estimated much less precisely with the coefficients of variation ranging from 5 to 10%, and some values exceeding 50%. Much of this variation was due to the variable contribution of the cholesteryl myristate peak as well as an incomplete resolution of the long-chain diacylglycerols and ceramides emerging in this region of the chromatogram. The values derived for total diacylglycerols and ceramides on the basis of calculations from the more precisely measured peaks, as de-

TABLE IV

REPRODUCIBILITY OF QUANTITATIVE ESTIMATES OF INDIVIDUAL CARBON NUMBERS ON REPEAT ANALYSIS OF NORMOLIPEMIC PLASMA SAMPLES

Values are given in mg % (mean ± S.D. of quadruplicate analyses).

Plasma samples	Carbon numbers of diacylglycerols and ceramides						Total phospholipids*	
	34	36	38	40	41	42	PC	SPH
1	9.27±0.03	36.16±0.08	38.38±0.23	13.76±0.17	8.77±0.08	4.12±0.16	106.6	23.8
2	10.49±0.04	42.13±0.10	39.93±0.09	12.18±0.09	11.07±0.06	3.84±0.04	117.1	26.7
3	10.62±0.25	48.01±1.95	40.24±2.03	12.99±1.09	11.40±1.21	4.34±0.74	127.2	26.1
4	10.28±0.03	42.63±0.12	43.17±0.10	14.11±0.10	10.68±0.07	4.31±0.07	123.4	25.9
5	9.67±0.05	39.05±0.17	40.81±0.15	14.61±0.16	10.21±0.05	4.06±0.34	114.6	24.6
6	9.24±0.09	39.06±0.21	39.06±0.10	14.02±0.18	8.35±0.05	3.85±0.21	112.5	23.2
7	8.78±0.10	37.75±0.26	34.70±0.23	10.98±0.14	8.85±0.07	3.49±0.51	104.2	21.9
8	7.48±0.23	33.40±0.12	34.17±0.57	11.49±2.97	7.67±0.14	4.69±0.05	98.1	18.5
9	8.37±0.11	37.93±0.08	38.85±0.46	15.24±0.12	8.15±0.12	4.40±0.14	111.7	20.6
10	9.20±0.19	45.46±0.21	43.12±0.16	15.66±0.46	8.71±1.23	4.95±1.29	129.7	22.0
11	7.06±0.11	34.37±0.07	34.46±1.01	15.87±0.02	6.21±3.23	4.10±1.10	100.9	17.0
12	8.74±0.47	39.71±0.09	42.26±0.11	15.46±3.79	8.64±0.96	4.94±0.39	119.5	21.5
13	9.30±0.34	45.56±0.06	45.08±0.11	16.00±0.09	8.74±0.13	4.66±0.06	132.9	22.3
14	8.52±0.57	37.60±0.22	39.80±0.78	16.68±0.33	8.18±0.09	4.59±0.45	112.4	21.1
15	8.16±0.53	37.81±0.24	37.65±0.18	12.44±3.80	4.97±3.23	3.28±1.71	109.4	19.9
16	7.84±0.68	35.78±1.40	36.94±2.24	14.03±1.22	6.63±2.65	3.51±2.02	105.9	19.2
17	7.63±0.54	38.30±0.23	40.39±0.86	11.13±3.32	7.59±0.60	3.07±0.49	116.4	18.2
Average							114.3±9.9	21.9±2.9

*Total phosphatidylcholine and sphingomyelin estimated as explained in the text.

scribed under Methods, show coefficients of variation averaging about 1% and 2%, respectively. The estimates for total phosphatidylcholine (114.3±9.9 mg%) and sphingomyelin (21.9±2.9 mg%) are significantly lower than the values (122.6 and 33.9 mg%, respectively) calculated from the data reported by Noel et al. [22] for these phospholipids from a much older population.

Table V gives the reproducibility of a decaplicate estimation of the peak

TABLE V

REPRODUCIBILITY OF QUANTITATIVE ESTIMATES OF INDIVIDUAL CARBON NUMBERS ON REPEAT PROCESSING OF PLASMA SAMPLES OF INCREASING LIPID CONTENT

Values are given in mg % (mean ± S.D. of decaplicates). A and B represent replicate GLC analyses only.

Plasma samples*	Ceramide and diacylglycerol carbon numbers			Total phospholipids**	
	34	36	38	PC	SPH
LRC 1A	13.51±0.19	50.75±0.75	54.68±0.82	150.089±2.34	34.93±0.54
LRC 1B	13.40±0.59	49.60±2.67	53.22±2.83	147.59 ±6.62	34.95±1.53
LRC 2A	17.78±0.60	59.38±1.97	64.22±4.29	172.58 ±8.15	47.40±1.91
LRC 2B	16.93±0.33	59.22±0.81	62.66±0.78	171.58 ±2.19	44.32±1.16
LRC 3	24.46±0.81	74.22±1.45	78.38±1.18	209.50 ±3.06	66.17±2.37

*LRC 1—3 represent control samples prepared by adding known amounts of VLDL or LDL to a common plasma pool.

**Total phospholipid estimated as explained in the text.

areas of carbon numbers C_{34}—C_{38} in a series of control pools prepared by adding VLDL or LDL to a common plasma pool to yield samples of increasing total lipid content. Each replicate constituted an independent digestion, extraction, dilution with the internal standard and derivatization of an aliquot of the plasma. It is seen that the precision of estimation of the completely resolved peaks is again very good. The coefficients of variation for the estimates of total phosphatidylcholine and sphingomyelin range from 1 to 4%. Furthermore, the values derived for these phospholipids by GLC for the various concentration levels compare closely to those expected from the knowledge of the composition of the lipoprotein fractions combined to obtain the synthetic plasma samples [Breckenridge (1975), unpublished results].

Fig. 4 shows a series of plots obtained for the estimates of the various carbon numbers of ceramides and diacylglycerols in a total of 137 A and B pairs of plasma samples analyzed over a period of one year without the knowledge of the sample identity. The correlation coefficients between the diacylglycerol peaks are: C_{36}, 0.95; C_{38}, 0.96; C_{40}, 0.96; C_{41}, 0.93 (103 pairs of data only); and C_{42}, 0.97 (33 pairs of data only). The correlation is best for the larger and more completely resolved peaks. The reproducibility is especially high for the diacylglycerol peaks C_{36} and C_{38}, which are used in the indirect estimation of the total phosphatidylcholine content. However, the correlation coefficient for the ceramide peak C_{34} used in the computation of total sphingomyelin content is also satisfactory (r = 0.88). The legend to Fig. 4 gives the slopes and intercepts of each regression line. In general these values are of the same order as

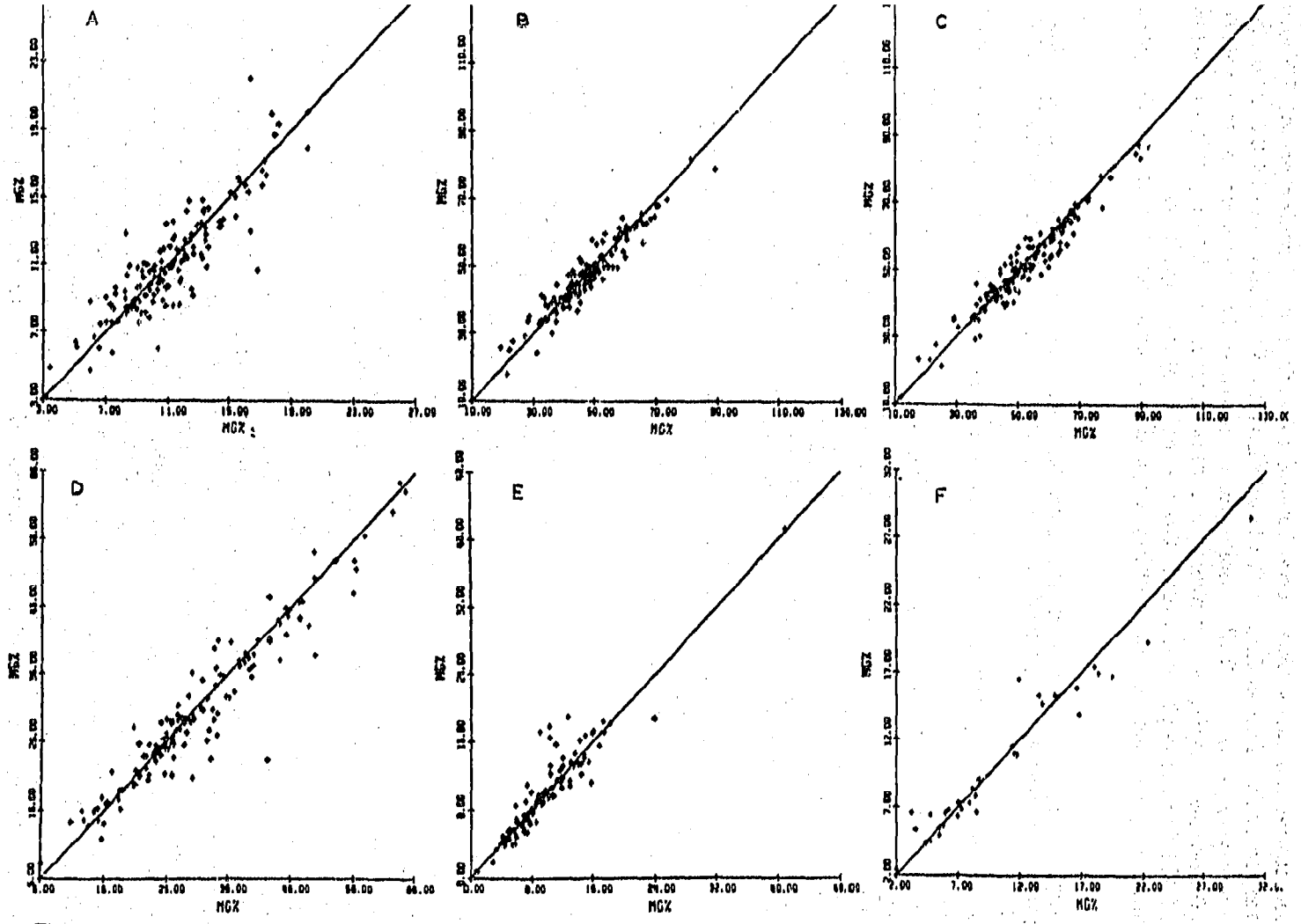

Fig 4. Paired comparisons of results (mg%) obtained by GLC for duplicate samples of plasma diacylglycerol and ceramide moieties of phospholipids over a period of two years. A, Peak 34 (slope = 0.88; intercept = 1.29); B, peak 36 (slope = 0.89; intercept 5.41); C, peak 38 (slope = 0.89; intercept 5.19); D, peak 40 (slope = 0.91; intercept = 2.09); E, peak 41 (slope = 1.13; intercept = −3.30); F, peak 42 (slope = 0.86; intercept = 1.34)

those recorded for replicate analyses of the same sample over short periods of time.

Fig. 5 shows the paired comparisons for the total phosphatidylcholine (PC) and sphingomyelin (SPH) content as calculated from the C_{36} + C_{38} and C_{34} peaks, respectively. Again excellent correspondence is obtained between the independent GLC analyses carried out on stored samples over a period of one year. The correlation coefficient (r) for the phosphatidylcholine is 0.96 and for sphingomyelin it is 0.88. The latter value is the same as that for peak C_{34} from which the sphingomyelin values were calculated. A fair correlation is also observed between the ratios PC:SPH in the paired samples, $r = 0.80$. The legend to Fig. 5 gives the actual slopes of the various linear relationships along with the corresponding intercepts. The correlations of the C_{40}—C_{42} peaks to each other

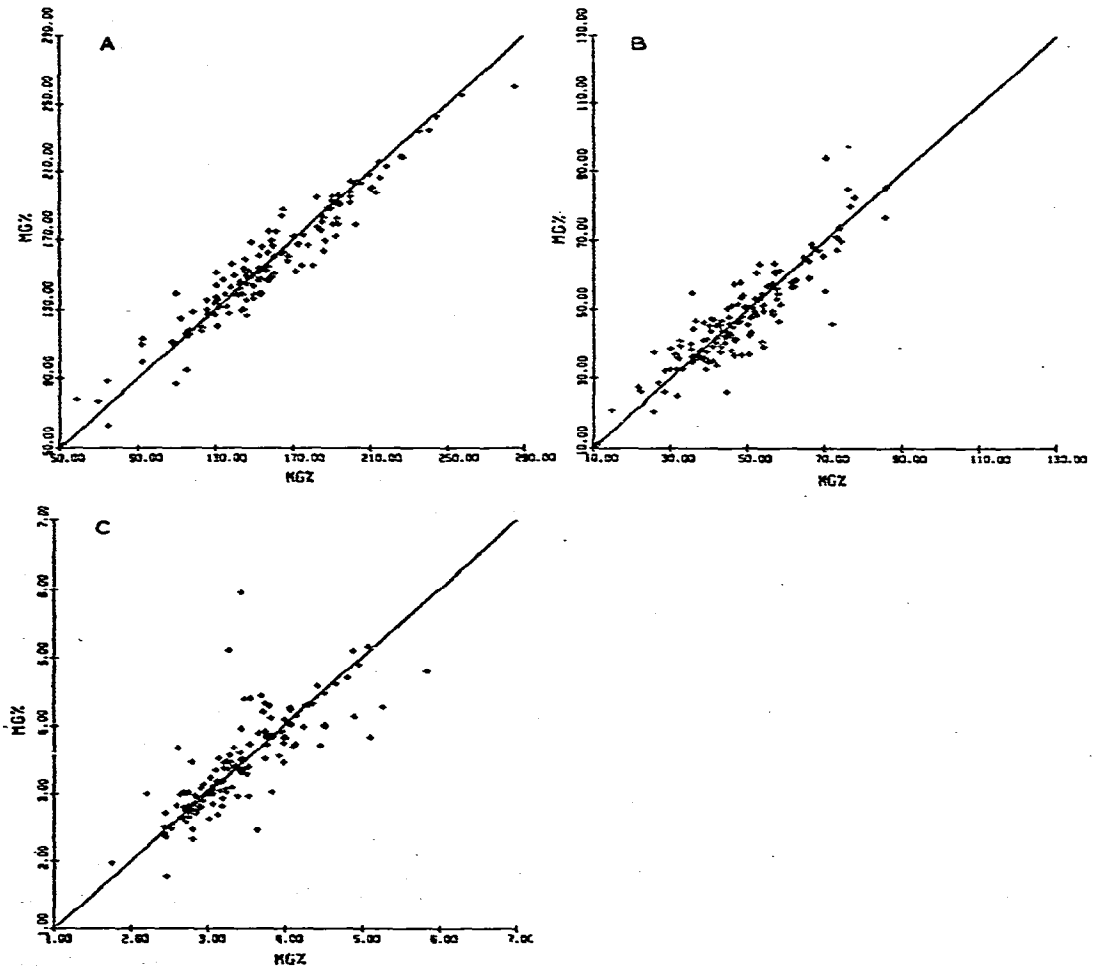

Fig. 5. Paired comparisons of results (mg%) obtained by GLC for duplicate samples of diacylglycerol and ceramide moieties of plasma phospholipids over a period of two years. A, PC (slope = 0.89; intercept = 16.33); B, SPH (slope = 0.82; intercept = 5.17); C, PC:SPH (slope = 0.86; intercept = 0.73).

and to the total phosphatidylcholine values were much poorer and indicated that the latter peak areas were not separately recorded in a manner sufficiently reproducible for a precise measurement of the molecular species of the phospholipids in these plasma samples. Poor correlations were also obtained for the levels of the lysophosphatidylcholines calculated from the total monoacylglycerol content in the A and B pairs of plasma samples (results not shown).

Comparative studies with manual phosphorus (TLC) methods

Table VI gives the statistical results of parallel analyses of 58 samples of

TABLE VI

COMPARISON OF GLC AND TLC ANALYSIS OF PHOSPHOLIPIDS IN WHOLE PLASMA AND ISOLATED LIPOPROTEIN CLASSES

Values are given as mean ± S.D.

Lipid classes*	No. of samples (*n*)	Automated GLC (mg %)	Manual phosphorus (mg %)
Total plasma			
PC	58	176.7±84.1	165.2±80.9
SPH	58	46.1±14.4	47.0±17.0
Total	58	222.8±92.4	212.3±94.6
PC:SPH	58	3.9± 1.27	3.5± 0.83
VLDL, LDL, HDL			
PC	23	70.9±30.1	72.9±29.7
SPH	23	18.9±11.6	23.9±12.0
Total	23	89.8±36.9	96.9±37.0
PC:SPH	23	5.24± 3.24	3.6± 1.7

*Abbreviations as given in the text.

whole plasma and 23 samples of VLDL, LDL and HDL_3 subfractions of it by the GLC and TLC methods. The values are generally in good agreement. Thus, the average value derived for total plasma phosphatidylcholine for the entire population was 177 mg% (GLC) and 165 mg% (TLC), while the corresponding values for sphingomyelin were 46 mg% and 47 mg%. The somewhat lower TLC value for phosphatidylcholine is due to a partial hydrolysis of the phosphatidylcholines upon storage of the plasma samples, an essentially complete agreement being obtained for the sum of the phosphatidyl and lysophosphatidylcholines by the two methods. Furthermore, the estimates for the total plasma phospholipids of both normolipemic and hyperlipemic subjects obtained by the GLC method corresponded to the range calculated from the data reported by Phillips and Dodge [23]; for example, 141—182 mg% (phosphatidylcholine) and 36—38 mg% (sphingomyelin) for a normolipemic population of a corresponding age, assuming molecular weights of 783 and 740, respectively, for the two phospholipid classes.

However, part of the difference is also due to the possible inclusion in the estimates for phosphatidylcholine of the estimates for phosphatidylethanolamine, which is present in plasma to about 4% of the total lipid phosphorus [19].

Fig. 6 shows a series of plots correlating the estimates for plasma phospha-

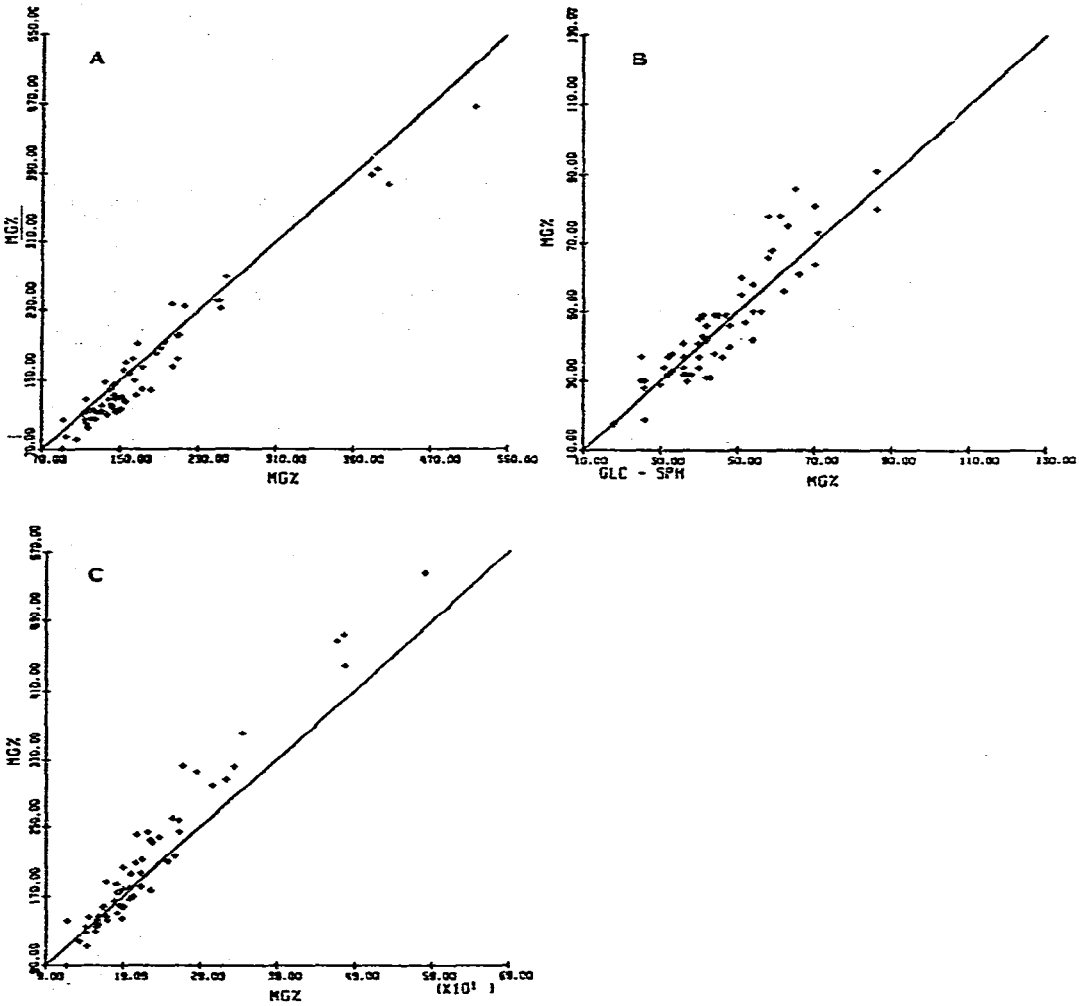

Fig. 6. Comparison of results (mg%) obtained by GLC (ordinate) and manual phosphorus (TLC) (abscissa) methods for whole plasma from subjects with a wide range of total lipids. A, PC (slope = 0.94; intercept = —1.01); B, SPH (slope = 1.06; intercept = —1.91); C, total (slope = 1.00; intercept = —10.04).

tidylcholines, sphingomyelin and total phospholipids as derived by the GLC and the manual phosphorus methods. For this purpose the phosphorus determinations were made on the individual phospholipid classes separated from plasma total lipid extracts by TLC, while the GLC estimates of the choline-containing phospholipids are based on the quantitation of the C_{36} + C_{38} and C_{34} peaks, representing the diacylglycerol and ceramide moieties, respectively. The estimates are derived from parallel analyses of a total of 62 samples of normolipemic and hyperlipemic plasmas. An excellent correlation is obtained for the phosphatidylcholine (r = 0.98) and for total plasma phospholipids (r = 0.98), with only slightly less effective agreement between the sphingomyelin analyses (r = 0.90). These correlations are somewhat inferior to those realized for du-

TABLE VII

COMPARISON OF ESTIMATES FOR PLASMA LYSOPHOSPHATIDYLCHOLINE AS OBTAINED BY GLC AND MANUAL PHOSPHORUS (TLC) METHODS

Plasma samples	GLC				TLC			
	LPC* (mg%)	PC (mg%)	LPC+PC (mg%)	LPCx100/(LPC+PC)	LPC* (mg%)	PC (mg%)	LPC+PC (mg%)	LPCx100/(LPC+PC)
Normal								
1	9	144	153.2	5.8	18.2	144.7	162.9	11.1
2	12	262	274.3	4.3	22.6	218.5	241.1	9.3
3	30	104	134.2	22.3	39.9	114.3	152.2	26.2
4	12	121	133.1	9.0	21.5	105.8	127.4	16.9
5	12	135	147.6	8.1	23.6	112.2	135.8	17.4
6	8	132	149.2	5.0	17.3	114.9	132.2	13.1
7	6	116	121.7	4.9	11.2	100.9	112.2	10.0
8	9	132	141.3	6.3	15.5	120.8	136.3	11.3
9	9	124	133.6	6.7	14.7	106.3	121.1	12.1
10	9	156	165.1	6.2	17.0	124.9	141.9	11.9
11	9	142	151.5	5.9	18.0	122.3	140.4	12.8
12	12	231	243.4	4.9	19.8	201.9	221.7	8.9
13	12	137	148.9	8.0	19.9	110.6	130.5	15.2
14	9	167	176.7	5.1	14.6	132.9	147.5	9.8
15	12	149	161.1	7.4	21.7	116.1	137.9	15.8
16	9	118	127.1	7.1	19.4	96.3	115.7	16.7
17	12	139	151.6	7.9	24.4	127.6	152.0	16.0
18	9	142	151.7	5.9	19.7	118.7	138.3	14.2
19	8	146	153.8	4.8	16.5	128.4	144.9	11.4
20	8	182	189.6	4.0	17.5	139.2	156.7	11.1
21	6	151	157.1	3.8	14.1	116.8	130.9	10.8
22	9	165	174.4	5.2	19.4	150.3	169.7	11.4
23	12	146	158.3	7.6	16.0	113.6	129.2	5.8
24	12	145	147.8	8.1	24.9	128.6	153.5	16.2
25	23	106	128.3	17.5	31.2	82.1	113.3	27.5
26	12	161	172.9	6.9	24.3	132.7	157.0	15.4
27	8	146	153.9	4.9	18.0	131.8	149.8	12.0
28	5	115	119.5	3.7	7.9	104.3	112.3	7.0

*Hyperlipemic***								
1	15	192	207	7.2	21.8	171.2	193	11.2
2	12	193	205	5.8	17.9	176	193.9	9.2
3	8	216	223.5	3.3	15.9	189.1	204.9	7.7
4	12	249	251	4.7	23.5	242.0	265.6	8.8
5	12	281	293	4.0	20.5	242.3	262.9	7.8
6	9	237	246	3.6	18.7	237.2	255.9	7.3
7	15	259	274	5.5	28.5	269.8	298.3	9.5
8	9	211	220	4.0	15.5	203.3	218.8	7.1
9	14	209	222.5	6.1	13.8	182.9	196.8	7.0
10	30	409	439	6.8	23.8	390.2	414.0	5.7
11	23	253	275.3	8.1	19.9	235.3	255.2	7.8
12	15	473	485	3.1	23.4	371.5	395.0	5.9
13	8	195	202.5	3.7	13.8	165.8	179.6	7.7
14	12	204	216	5.5	12.7	166.3	178.9	7.1
15	27	517	544	5.0	23.9	468.8	492.7	4.8
16	14	209	222.5	6.1	11.6	176.1	187.7	6.2
17	14	415	428.5	3.1	19.7	397.3	416.9	4.7
18	31	427	458	6.7	24.5	378.5	403	6.1
*Intralipid****								
1	8	227	234.5	3.2	16.2	194.5	210.7	7.7
2	6	105	111	5.4	11.0	115.1	126.1	8.7
3	3	104	107	2.8	5.9	70.4	76.4	7.8
4	12	253	265	4.5	18.9	232.6	251.5	7.5

*LPC, sum of peaks 22 and 24; other lipid classes estimated as described in the text.
**Hyperlipemic samples as in Table II.
***Fasting samples of plasma 16 h after receiving moderate doses of Intralipid.

plicate analyses of the same sample by either method alone. The good agreement between the estimated and measured levels of plasma phosphatidylcholine indicates that the sum of C_{36} + C_{38} peaks represents an essentially constant proportion (81±1%) of total species of plasma phosphatidylcholine on normal diets, as already noted in Table I. The estimation of the total plasma sphingomyelin on the basis of the single ceramide peak is somewhat less effective. This is due largely to the greater scatter of the values arising from a measurement of a relatively small peak area in the total GLC profile. When sufficiently large area is recorded for the ceramide peak (C_{34}), a more precise and an apparently accurate account of the total ceramide or sphingomyelin content is obtained because this peak constitutes an essentially constant proportion (30%) of the total plasma sphingomyelin under normal dietary conditions, even though the total amount of sphingomyelin in the plasma may vary.

Table VII compares the estimates for plasma lysophosphatidylcholine derived by the phospholipase C—GLC and the manual phosphorus—TLC methods of analysis. It would appear that the GLC method underestimates the lysophosphatidylcholine level by about 50%. The correlation coefficient for the two estimates is also relatively poor (r = 0.69). Since the GLC analyses of the samples were performed on fresh samples while the phosphorus analyses were done on samples that had been stored for 1—2 years, it is likely that the discrepancies are due to variable hydrolysis of the phosphatidylcholine during storage. This possibility is supported by the observation that the sum of phosphatidylcholine and lysophosphatidylcholine showed excellent agreement between the two methods (r = 0.98; slope = 1.08; intercept = 1.57). Analyses of samples performed by both methods at the same time gave comparable results (data not shown). Furthermore, when appropriately calibrated, the GLC estimates of the lysophosphatidylcholine levels were within the range (5—10% of the total lipid phosphorus) reported in the literature [19, 22], while those obtained by the phosphorus analysis on the stored samples were significantly higher. There is a possibility, however, that the GLC values could have been lower due to an incomplete digestion of the plasma lysophosphatidylcholine by phospholipase C [24, 25]. The possibility that much of the discrepancy between the two methods was due to partial hydrolysis during the time intervening between the analyses is further attested to by the relatively good agreement between the values of lysophosphatidylcholine expressed as percentages of the sum of lysophosphatidylcholine plus phosphatidylcholine measured by the two methods (r = 0.81).

The lysophosphatidylcholines were largely absent from the plasma lipoprotein fractions prepared in the present experiments as examined by both methods. Previous studies [19, 22], however, had reported small and variable amounts of lysophosphatidylcholine in various preparations of HDL, which, however, could have been due to contamination with albumin and/or lipoproteins of higher density. It is therefore obvious that relatively accurate estimates of plasma phosphatidylcholine may be obtained by direct GLC analysis when appropriate periods of enzyme digestion are selected along with appropriate correction and calibration factors of peak areas, as claimed previously on the basis of much more limited data [2].

Fig. 7 shows a series of plots correlating the results of some 69 parallel anal-

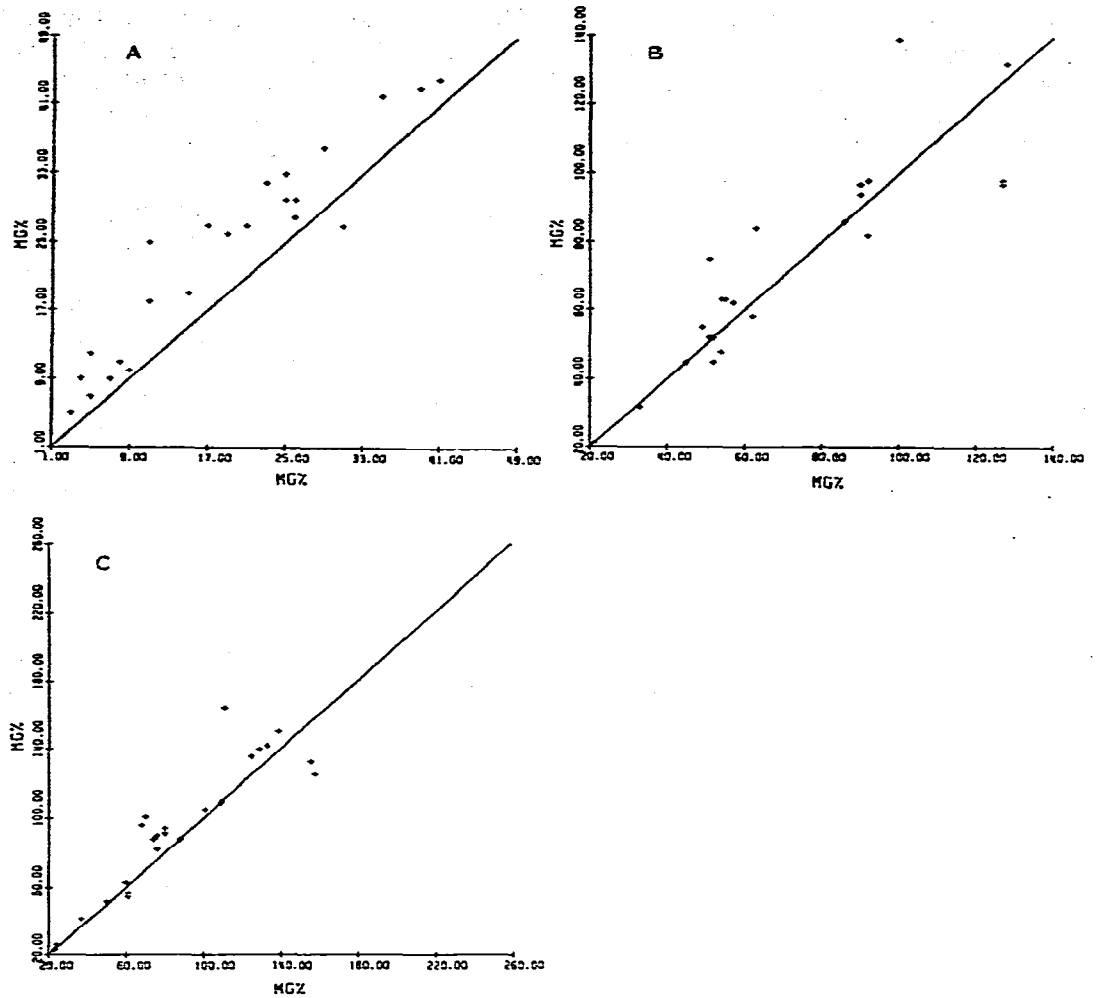

Fig. 7. Comparison of results (mg%) obtained by GLC (ordinate) and manual phosphorus (TLC) (abscissa) methods for plasma lipoproteins from subjects with a wide range of total lipids. A, SPH of VLDL, LDL and HDL (slope = 0.98; intercept = 5.29); B, PC of VLDL, LDL and HDL (slope = 0.87; intercept = 11.47); C, total PL of VLDL, LDL and HDL (slope = 0.90; intercept = 16.18).

yses of the phosphatidylcholine and sphingomyelin content of isolated plasma lipoproteins. Because of the relatively small number of parallel analyses made on the individual lipoprotein classes, the values for the different comparisons have been plotted on the same graph. Again the GLC estimates of the phosphatidylcholines are based on the total amount and relative proportions of peaks C_{36} and C_{38} for diacylglycerols and of peak C_{34} for ceramides. As can be seen the agreement is reasonably close for the phosphatidylcholines (r = 0.88) and total choline-containing phospholipids (r = 0.89), with the discrepancies approximating the reproducibility of duplicate estimates for the same sample with either method alone, when executed at a comparably low level of sample concentration. Surprisingly, the correlation was better for the estimates of the

sphingomyelin of the lipoproteins ($r = 0.95$). The linear regression plot for the phosphatidylcholines gave a slope of 0.87 with an intercept value of 11.47. The corresponding slope for the sphingomyelins was essentially unity (0.98) with an intercept value of 5.29, which represented a significant underestimation of this phospholipid by the GLC method, or an overestimation by the manual phosphorus method. The slope and intercept values for the total phospholipid comparison in the VLDL, LDL and HDL fractions were 0.90 and 16.18, respectively. An examination of the limited number of parallel comparisons carried out with the individual lipoprotein classes revealed that the best correlations were obtained for the phosphatidylcholines of the VLDL and LDL fractions and for the sphingomyelins of the LDL fraction. The correlations for the sphingomyelins of the VLDL and HDL fractions and the phosphatidylcholines of the HDL fractions were less perfect, but there was no obvious explanation for the differences in the estimates obtained by the two methods. Both methods were clearly measuring the same components with about the same precision. It should be noted that the GLC estimates for total plasma sphingomyelins were in good general agreement with those obtained by the manual phosphorus method (Table VI, Fig. 6). Obviously, the GLC method can be used for a reliable estimation of both the total choline-containing phospholipids of plasma, and of the individual phosphatidylcholine and sphingomyelin classes. The agreement between the manual phosphorus method and the automated GLC technique could be further improved by including in the GLC estimates also the small amounts of diacylglycerols likely to be released by phospholipase C from plasma phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol [19]. These contributions, however, are uncertain at the moment because of the lack of knowledge about the molecular species composition of the minor phospholipids of plasma. Theoretically a correction should also have been made for the free plasma diacylglycerols, the molecular species of which were known. These corrections, however, would not have seriously affected the correlations described.

There was very little free monoacylglycerol or lysophosphatidylcholine associated with the major plasma lipoproteins, as they presumably remained in the $d < 1.21$ fraction in complex with albumin [19].

DISCUSSION

We had previously shown that either pyrolysis [1] or hydrolysis with phospholipase C [2] results in a reproducible conversion of the phospholipids to neutral lipids to permit a GLC estimation of the sum of the diacylglycerophospholipids and sphingomyelins of plasma along with the free cholesterol, cholesteryl esters and triacylglycerols. The present study confirms these observations and also demonstrates that precise and accurate estimates may separately be obtained for the phosphatidylcholines and sphingomyelins provided certain peak areas are quantitatively measured and certain assumptions about the composition of the molecular species of the plasma phospholipids are valid. Although we have now documented the major assumptions and extrapolations, there is a need for a further discussion of this part of the work.

Validity of analytical basis

The present GLC estimates of the phosphatidylcholine and sphingomyelin content of whole plasma and of the individual lipoprotein classes is based upon a reproducible separation, identification and quantitation of selected molecular species of diacylglycerols and ceramides released from these phospholipids by phospholipase C. The selected reaction conditions result in an essentially complete hydrolysis of the plasma phosphatidylcholines and sphingomyelins. A TLC examination of the reaction products has failed to yield any measurable phosphorus for the corresponding undigested phospholipids. The hydrolysis of standard lysophosphatidylcholine was about 90% complete, while the other minor plasma diacylglycerophospholipids (ethanolamine, serine and inositol phosphatides) were hydrolyzed to about 20—25% of their content. These findings are supported by previous reports in the literature which have indicated a comparable extent of hydrolysis of these phospholipids either separately or in mixture with other phospholipids [26—28].

The range of the carbon numbers of the diacylglycerols released from the plasma phosphatidylcholines remains the same on normal diets, although the proportions of the individual carbon numbers change with the source of the dietary fat. These changes in the diacylglycerol proportions are confined essentially to carbon numbers C_{36} and C_{38} which together still account for 81±1% of the total. Since the latter peak areas can be measured with great precision under all experimental conditions, it is possible and practical to use them to estimate the total phosphatidylcholine content of the plasma or any lipoprotein fraction, when estimates of individual molecular species are not necessary. There are no known changes in the carbon numbers of the ceramides with diet. It is therefore practical to use the carbon number C_{34}, which accounts for 30±1% of the total sphingomyelin, as a reliable indicator of the content of spingomyelin in total plasma. Because of the change in the carbon number proportions among the lipoproteins, the sphingomyelin content of HDL_3 must be estimated by assigning 24±1% of the total peak area to peak C_{34}. The finding that the HDL_3 sphingomyelins have a somewhat higher molecular weight than those of other lipoproteins of plasma is in agreement with earlier observations on their fatty acid composition [19], although from different subjects. We have recently confirmed these findings in samples collected from the same subjects [20].

Recoveries of plasma phospholipids

The relative recoveries of the diacylglycerols and ceramides in the GLC profiles were of the order anticipated on the basis of the known proportion of the corresponding phospholipids in human plasma [19, 23]. The absolute recoveries of the phospholipids from whole plasma by the GLC method were calculated by comparison with the values obtained by the manual determination of the phosphorus in the various phospholipid fractions isolated by TLC from the corresponding plasma samples. It was seen that the means and standard deviations for total phospholipids, total phosphatidylcholine and sphingomyelin corresponded closely, as did the phosphatidylcholine:sphingomyelin ratios. The TLC method gave somewhat lower values for phosphatidylcholine and hence total phospholipid because of the partial hydrolysis of the phosphatidylcholine

upon storage of plasma samples. Correlation plots of the values obtained by the GLC procedure and the manual phosphorus analysis method showed points falling on both sides of the ideal correlation line with about equal frequency. The average values obtained for total plasma lysophosphatidylcholine by the GLC method (4 mg%) when corrected for the presence of about 8 mg% of free monoacylglycerol, were only slightly below the range (5—8 mg%) calculated from the data reported by Phillips and Dodge [23], assuming a molecular weight of 524. The values measured by TLC on the stored samples averaged 19 mg%. Clearly, the GLC estimates for plasma phosphatidylcholine and sphingomyelin are in the range previously reported by different laboratories [19, 22, 23], while the estimates for the lysophosphatidylcholine are somewhat lower due to incomplete hydrolysis by phospholipase C [24, 25] and because of possible incomplete extraction of the released monoacylglycerols [29].

Recovery of lipoprotein phospholipids

Likewise, the relative recoveries of the diacylglycerols and ceramides in the GLC profiles of the lipoproteins were of the order anticipated for the corresponding phospholipids in the appropriate fractions of human plasma [19, 22]. The average absolute values derived from specific paired comparisons showed good general agreement, comparable to that obtained for total plasma lipid analyses, while the correlation plots of the values obtained by the two methods showed points falling on both sides of the ideal correlation line with nearly equal frequency. An exception was provided by the HDL fraction, which failed to yield truly identical estimates by the two methods for sphingomyelin. It is possible that higher values derived by the phosphorus analysis for the HDL sphingomyelin were due to some lipid phosphorus compound which overlapped with sphingomyelin in the TLC system employed. Alternatively, some species of sphingomyelin in the HDL fraction may have resisted either the digestion with phospholipase C or solvent extraction to a greater extent than those in the other lipoproteins. The various lipoprotein fractions examined in the present study were essentially free of lysophosphatidylcholine, but small amounts of this minor phospholipid have been reported in plasma lipoprotein fractions prepared by other laboratories [19, 22].

Influence of neutral lipids on GLC quantitation of phospholipids

The relative and absolute recoveries of the different phospholipid classes are influenced to a variable although minor extent by the presence of neutral glycerolipids and low-molecular-weight cholesteryl esters in the total lipid extracts of whole plasma or of the lipoprotein fractions. We have estimated that the free plasma diacylglycerol content ranges from 1 to 2% of the total neutral lipid, which corresponds to about 4% of the total plasma lipid as claimed by Skipski et al. [30]. Furthermore, the content of free diacylglycerols was found to be proportional to the total triacylglycerol content of the sample, including lipoprotein fractions. However, increased plasma triacylglycerol levels were usually associated with increased amounts of phosphatidylcholine, so that the relative contribution of free diacylglycerols to the diacylglycerols arising from hydrolysis of phosphatidylcholine remained about the same in all instances ($< 2\%$). Furthermore, since the free diacylglycerols were made up of at least two major

carbon numbers (C_{36} and C_{38}) of about equal proportions, they contributed less than 2% to the total peak area of the corresponding diacylglycerols derived from the plasma phosphatidylcholine. The plasma free ceramide levels (0.4% of total) were too low to affect significantly the estimates of sphingomyelin based on the release of the bound ceramides by phospholipase C.

The contamination of the longer chain diacylglycerol and ceramide peaks with the peaks due to the short-chain cholesteryl esters (largely cholesteryl myristate) was much more significant and was mainly responsible for the poor reproducibility of the C_{40}—C_{42} peak areas measured from the retention time window. The overlap was especially bad in samples with high proportions of cholesteryl esters, when shoulders or poorly defined peaks with flat tops were recorded. As a result it was frequently impossible to measure the contributions of the C_{40}—C_{42} peaks to the total lipid profile during the fast programming rates. It was therefore necessary to calculate the total diacylglycerol and ceramide peak area from precise measurements of a few peaks as explained above. This difficulty may be eliminated or minimized by using capillary GLC columns [J.J. Myher and A. Kuksis (1979), unpublished results].

The free plasma monoacylglycerols represent largely C_{16} and C_{18} fatty acid esters just like those that are released from plasma lysophosphatidylcholines by digestion with phospholipase C. As a result there may occur a significant overestimation of the plasma lysophosphatidylcholine levels by the GLC method unless correction is made for the content of free monoacylglycerols. Although apparently correct estimates for lysophosphatidylcholine can be obtained, this correction is not entirely sound because of the frequent appearance of other unknown lipid components in this part of the plasma lipid profile. Since both free monoacylglycerols and lysophosphatidylcholines are largely absent from the plasma lipoproteins, the quantitation of these components has not been further pursued.

Despite the limitations discussed above, this study shows that with precise peak area integration the GLC method can provide plasma phospholipid estimates approaching those obtained by direct measurements of phosphorus on isolated phospholipid classes. A statistical evaluation of the data demonstrates that the quantitative GLC estimates for total plasma and lipoprotein phosphatidylcholines and sphingomyelins are of about the same order of accuracy and precision as those demonstrated for the plasma cholesterol and triacylglycerols [9]. The GLC analyses of phospholipids are recommended in conjunction with plasma neutral lipid analysis, which results in a more efficient utilization of the required specialized equipment and analytical expertise.

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