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ANALYSIS OF LONG-CHAIN ACIDS OF HUMAN PLASMA PHOSPHATIDYLCHOLINES (LECITHINS) AND CHOLESTERYL ESTERS BY GLASS OPEN TUBULAR CAPILLARY COLUMN GAS CHROMATOGRAPHY FOR STROKE PATIENTS AND FOR NORMAL SUBJECTS

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SUMMARY

Methods are described for long-chain acid compositional analyses of human plasma phosphatidylcholines and plasma cholesteryl esters. Conventional procedures were employed for the isolation of these lipids and for the preparation of methyl esters of the acids. The gas chromatographic analytical procedure was based upon the use of thermostable glass open tubular capillary columns with a new polar phase. The methods were used in a study of plasma lipids in stroke patients and in normal young adult subjects.

INTRODUCTION

Lipids present in human plasma include phosphatidylcholines (lecithins) and other phospholipids, cholesteryl esters, cholesterol, triglycerides, free fatty acids, and α-tocopherol. In a previous paper[1], a new gas chromatographic (GC) procedure suitable for the concurrent determination of free fatty acids and α-tocopherol in plasma was described. This was based upon use of capillary columns with the Schwartz–Mathews liquid phase[2] (a polyphenyl ether sulfone). The glass open tubular columns were prepared by the method of Lin *et al.*[3], and the gas chromatograph was modified to employ the injection system of German and Horning[4]. When this method was used in studies of plasma samples from stroke patients and from normal subjects, it was found that the patient group differed from the normal group in two respects. The α-tocopherol plasma concentration values were lower (below 0.5–0.6 mg/dl), and the linoleic acid to oleic acid ratio for the free fatty acid fraction was depressed.

These results suggested that compositional studies should be carried out for plasma phosphatidylcholines and plasma cholesteryl esters for the two groups of subjects. It was expected that a depressed linoleic acid to oleic acid ratio would be found for stroke patients, because of the depressed ratio found for free fatty acids. This view was in accordance with the results of Rosenlund *et al.*[5], who found that in children with cystic fibrosis both of these lipid classes showed depressed linoleic acid to oleic acid ratios (the average concentration of α-tocopherol in children with cystic fibrosis was found in a study of Underwood *et al.*[6] to be about 0.2 mg/dl).

A new analytical procedure for obtaining compositional data for plasma phosphatidylcholines and cholesteryl esters was employed. The initial steps were based on conventional methods, but the GC analyses were carried out with a thermostable glass open tubular capillary column of the type employed in the previous study[1]. The patient group showed uniformly depressed ratios of linoleic acid to oleic acid in plasma phosphatidylcholines, when compared with normal subjects. Many, but not all, patients showed a depressed ratio for cholesteryl esters. Some patients showed a marked elevation in arachidonic acid in plasma phosphatidylcholines.

These results permit the following hypothesis to be formulated. Thrombotic strokes are a consequence of platelet aggregation, caused by endoperoxide formation resulting from an increased substrate supply of arachidonic acid, and possibly aided by an abnormal phospholipid composition in plasma and platelets. This situation results from a sequence of events starting with a marginal increase in the rate of metabolism over the rate of absorption of α-tocopherol, leading to α-tocopherol depletion; the later steps include a decrease in incorporation of linoleic acid into phosphatidylcholines and an increase in substrate availability of arachidonic acid. It is not known if the effect of α-tocopherol depletion is specific or if another substance is involved.

EXPERIMENTAL

Extraction of lipids from plasma

Lipids were extracted according to the procedure of Folch *et al.*[7]. A measured volume of plasma, usually 0.5 ml, was placed in a separatory funnel; 100 ml of chloroform–methanol (2:1) and 10 ml of distilled water were added. The separatory funnel was shaken vigorously for 10 min, and then placed in a refrigerator (4°) overnight in order to allow complete phase separation. No difficulties with emulsion formation were encountered.

The lower lipid-containing phase was separated and concentrated with a rotary evaporator (40°). The sample was transferred with 3 ml of chloroform–methanol (2:1) to a 3.5-ml vial fitted with a PTFE-lined screw cap. The solvents were evaporated (room temperature, nitrogen stream). The residue was dissolved immediately in 0.5 ml of chloroform–methanol (2:1).

Thin-layer chromatography

Thin-layer chromatographic (TLC) separations were carried out with glass fiber paper impregnated with silicic acid (ChromAR 500; Mallinckrodt, St. Louis, Mo., U.S.A.). The solvent systems were: chloroform–methanol–water (65:25:4) for lecithins and hexane–chloroform (1:1) for cholesteryl esters. In order to obtain a complete separation of cholesteryl esters from triglycerides, the solvent system should be placed in the developing tank overnight.

After activating the ChromAR 500 sheets (20 × 20 cm) at 100° for 30 min, a 200-μl sample was applied as a band with a syringe. Each sheet was developed for 20 min. After drying in air for 50 sec, a 1-cm strip at both edges of the sheet was cut and exposed to iodine vapor. The fraction of interest was identified, and the appropriate strip was cut for the transesterification reaction. Alternatively, the entire sheet

was exposed very briefly. These steps were carried out rapidly to avoid excessive air exposure of dried films of lipids.

Transesterification

The band containing the lipid fraction was cut into small strips (2 × 8 mm); these were placed in a tube (15 ml) equipped with a PTFE-lined screw cap. Transesterification was carried out by adding 2 ml of methanol and 0.08 ml of concentrated sulfuric acid; the sample was heated at 60° for 3 h. Long-chain methyl esters were extracted by adding 2 ml of glass-distilled water and 4 ml of isooctane. The tube was shaken, centrifuged, and the supernatant solution was separated. The solvent was removed at room temperature with the aid of a nitrogen stream.

For phosphatidylcholine analyses, 30 μl of isooctane were added to the residue; 3 μl of this solution were used for chromatographic analysis. For cholesteryl esters, the cholesterol was derivatized by adding 25 μl of pyridine, 25 μl of bis(trimethylsilylacetamide) and 12.5 μl of trimethylchlorosilane to the residue. The mixture was heated at 90° for 1 h. The resulting solution containing both long-chain methyl esters and the trimethylsilyl ether of cholesterol was used directly for chromatographic analysis.

Gas chromatography

An F and M Model 400 gas chromatograph was modified to include the injection system of German and Horning[4], and to accept glass open tubular capillary columns. The conditions of operation were: injector temperature, 250°; detector temperature, 280°; temperature programming, 2°/min; carrier gas, nitrogen; initial column pressure, 5 p.s.i.; split ratio, 10:1. The picoammeter was a Keithley Model 417, in place of the original electrometer–amplifier.

The glass column used in this study was 32 m × 0.25 mm I.D.; it was drawn to provide a 12-cm-diameter coil. The coating of phase PZ-176[2] was applied as described earlier[3]. Calculations were made from hand measurements of peak height and width at half-height. Estimates of precision and accuracy are in an earlier paper[1].

Biological samples

Blood samples were obtained during studies of thrombotic stroke patients who required, for other purposes, the insertion of a catheter in the jugular vein. All samples were taken during a 10–20 day post-stroke period. Samples from normal subjects were taken from an antecubital vein with a disposable syringe. All samples were centrifuged within 15–20 min after collection.

RESULTS AND DISCUSSION

The objective of this work was to obtain long-chain acid compositional data for the phosphatidylcholines (lecithins) of human plasma in normal subjects and in stroke patients. It was also desired to obtain similar data for plasma cholesteryl esters, and to determine plasma α-tocopherol concentrations by the method described previously[1].

The usual procedures for compositional studies of plasma phosphatidylcholines and cholesteryl esters involve three steps: isolation of an appropriate lipid fraction

from plasma, separation of individual lipid classes by partition chromatography, and compositional analysis by GC of the long-chain esters obtained after transesterification. In this study, conventional procedures were used for the first two steps, and a new GC procedure was used for the third step.

A lipid fraction containing both phosphatidylcholines and cholesteryl esters was obtained from plasma samples by extraction with chloroform–methanol (2:1) according to the procedure of Folch *et al.*[7]. Class separations were carried out by partition chromatography with ChromAR sheets (silicic acid) under conditions approximating those used in TLC, and with visualization by exposure to iodine vapor. Separate sheets were used for the two class separations required for each sample. We prefer this form of chromatography instead of silicic acid column chromatography because of the speed of the separation. The chief problem is to avoid air oxidation of polyunsaturated long-chain acids. The usual precautions are to use a nitrogen atmosphere in all evaporation steps, to avoid complete drying of the sheets, and to carry out the transesterification reaction as soon as possible after the separation, in order to avoid prolonged exposure of thin films of the lipids to air. With these precautions, the lipid classes can be analyzed without loss of polyunsaturated acids. Transesterification was carried out in methanol–sulfuric acid instead of the more widely used methanol–boron trifluoride or methanol–hydrogen chloride mixtures. We prefer this procedure (with the addition of benzene when triglycerides are under study) since the required solutions can be freshly prepared for each reaction (the more usual method is to employ commercially prepared mixtures).

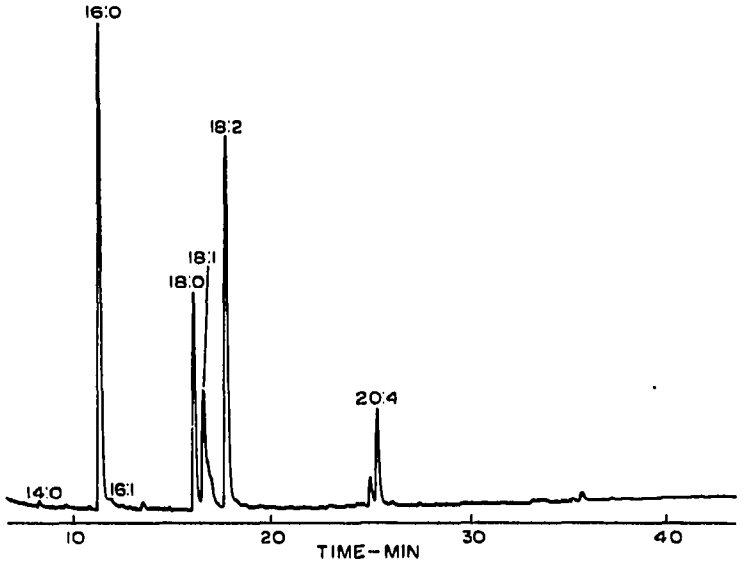

Fig. 1. GC analysis of long-chain methyl esters from plasma phosphatidylcholines for a normal adult. The conditions are described in Experimental. The major components, in order of elution, are the esters of palmitic acid, stearic acid, oleic acid, linoleic acid, and arachidonic acid. The presence of isomers in the oleic acid peak is evident. The substance accompanying arachidonic acid is a 20:3 acid identified earlier as 20:3ω6. The distribution of fatty acids (analyses are in Table I for all normal subjects) is not uniform for all individuals, but the same acids are always present. Data for all normal subjects are in Table I.

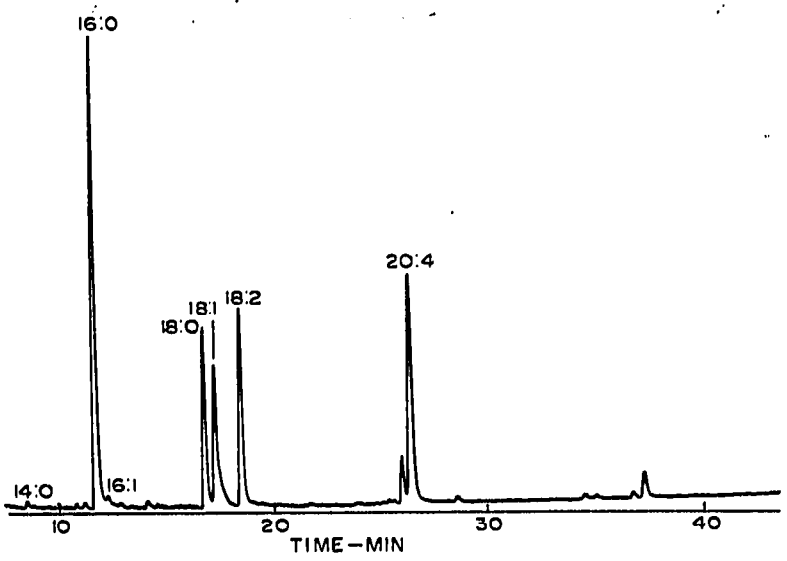

Fig. 2. GC analysis of long-chain methyl esters from plasma phosphatidylcholines for a stroke patient. Conditions, same as for Fig. 1. The major components, in order of elution, same as in Fig. 1. A marked change in the linoleic acid to oleic acid ratio is evident, and arachidonic acid is present in greater amount than linoleic acid. The relative amount of a minor component (a longer-chain polyunsaturated acid) is also increased; this compound, which is usually identified as 22:6ω3, was not included in the compositional analyses. Data for all stroke patients are in Table I.

Almost all compositional studies of long-chain acids in lipids are still carried out essentially by methods developed during 1960–1965. Packed-column GC with polar phases leads to separation of the methyl esters according to both chain length and degree of unsaturation. The chief problems are overlap of highly unsaturated

TABLE I

LONG-CHAIN ACID COMPOSITION OF PLASMA PHOSPHATIDYLCHOLINES OBSERVED FOR NORMAL SUBJECTS AND FOR STROKE PATIENTS

Acid						
16:0	*16:1*	*18:0*	*18:1*	*18:2*	*20:3**	*20:4*
*Normal subjects***						
38.5	0.3	17.5	8.9	24.3	1.6	8.8
37.7	—***	14.4	12.8	27.7	0.9	6.4
27.8	1.0	18.3	15.1	29.4	2.1	6.2
29.7	—	20.0	13.0	28.7	1.7	6.9
29.1	—	19.4	9.9	31.1	2.3	8.2
31.1	—	17.2	9.1	31.3	2.3	8.8
34.2	0.2	17.4	9.9	26.0	2.1	9.9
32.9	—	23.4	8.9	31.1	—	3.3
35.2	—	17.9	9.4	27.2	0.7	9.3
29.0	—	23.0	12.7	29.5	0.4	4.7
40.5	—	18.7	11.3	22.3	1.3	5.6
31.7	0.2	16.8	10.8	28.3	2.8	9.0
34.8	0.3	17.3	11.5	22.4	2.8	10.5

(Continued on p. 488)

TABLE I *(continued)*

Acid						
16:0	*16:1*	*18:0*	*18:1*	*18:2*	*20:3**	*20:4*
*Stroke patients***						
32.9	0.5	18.5	13.3	22.2	2.7	10.1
30.2	0.5	16.6	13.5	18.5	2.6	18.2
35.7	0.3	19.1	13.2	15.0	3.1	13.6
34.7	0.4	14.4	14.4	21.8	4.7	24.1
41.5	0.2	17.3	11.1	16.4	1.8	11.5
38.4	—	13.3	11.5	20.7	2.1	14.0
32.0	0.6	18.8	11.6	18.8	3.4	14.5
40.4	0.4	17.5	12.5	16.8	3.2	9.2
41.8	0.2	18.8	15.0	15.0	1.7	7.2
32.3	0.7	17.5	19.9	14.3	2.5	12.4
31.1	0.2	18.7	16.6	19.6	1.8	11.7
42.4	—	15.3	13.8	19.0	1.4	7.8
43.4	0.6	13.3	12.6	21.2	1.1	7.4
37.6	0.2	22.8	10.1	17.9	1.4	9.6
43.4	—	16.7	12.0	15.7	0.7	11.1
38.7	0.3	17.6	12.6	14.3	1.0	15.3
42.6	—	16.9	13.8	19.8	—	6.7
34.9	0.2	19.4	10.9	16.4	2.0	15.9
39.6	0.3	16.7	12.3	22.1	1.3	7.3

* This acid is 20:3ω6, according to earlier studies.

** These data are in general agreement with analyses carried out earlier (1961–1967). Nelson[18], working with a patient group with "above average serum lipid levels", reported (pooled plasma sample) an arachidonic acid concentration of 12.2%. The calculated linoleic acid to oleic acid ratio is 2.4; this fits the results found (Table III) for young adult normal subjects, but with a higher arachidonic acid value. Saturated acids (16:0 and 18:0) accounted for about 40% of the total acids; the present analyses show about 50% saturated acids. The study of Balint *et al.*[19] showed that phosphatidylcholines from bile and plasma were significantly different in composition, but that phosphatidylcholines from plasma samples taken at different sites in the body had substantially the same composition (except that phosphatidylcholines from arterial blood contained less palmitic acid than those from venous blood). The concentrations of oleic acid, linoleic acid and arachidonic acid found for phosphatidylcholines from a venous blood sample from a patient were 15.0, 27.4, and 15.1%, respectively. These values, because of the calculated linoleic acid to oleic acid ratio (1.82) and the arachidonic acid concentration (15.1%), correspond to values expected (Table III and Fig. 8) from our work for an individual at biochemical risk of thrombus formation. Saturated acids (16:0 and 18:0) constituted only 32% of the total acids. The compositional data reported by Phillips and Dodge[20] show average (six subjects) concentrations of 12.9, 24.7 and 10.9% for oleic acid, linoleic acid, and arachidonic acid, respectively. The calculated value for the linoleic acid to oleic acid ratio is 1.81, corresponding to the data of Balint *et al.*[19] but with a lower arachidonic acid concentration. Saturated acids constituted about 40% of the total fatty acids.

*** Below 0.2%.

esters of the same chain length with saturated or monounsaturated esters of the next longer chain length group, difficulties with column bleed when very long chain esters are under study, and failure to separate bond and *cis/trans* isomers of unsaturated esters. The use of capillary columns for the study of highly complex ester mixtures has been advocated, notably by Ackman[8], but suitable glass open tubular columns have not been available until very recently. In this work, the liquid phase was a thermostable polyphenyl ether sulfone synthesized by Mathews *et al.*[2], and the open tubular

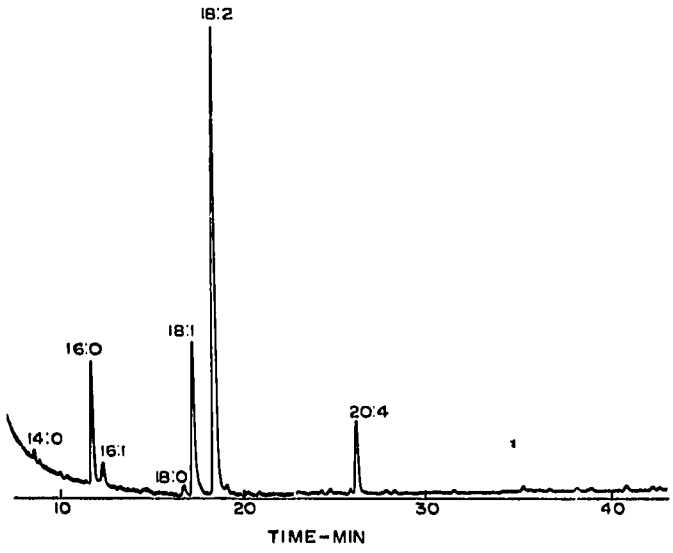

Fig. 3. GC analysis of the long-chain methyl esters from plasma cholesteryl esters of a normal adult. Conditions, same as for Fig. 1. The acids, in order of elution, are palmitic, palmitoleic, stearic, oleic, linoleic, and arachidonic. The usual linoleic acid to oleic acid ratio is about 3:1. Data for all normal subjects are in Table II.

columns were prepared by a process developed for use with polar phases[3]. The nature of the separations, and evidence supporting the use of these columns in quantitative analytical studies, was summarized in the first paper in this series[1].

Figs. 1 and 2 show examples of separations of the long-chain acids (as methyl esters) of human plasma phosphatidylcholines for a normal subject and a stroke patient. Table I contains compositional data for all individuals. Figs. 3 and 4 show examples of separations of the long-chain acids (as methyl esters) of plasma cholesteryl esters for a normal subject and a stroke patient. Table II contains the corresponding cholesteryl ester compositional data for all individuals.

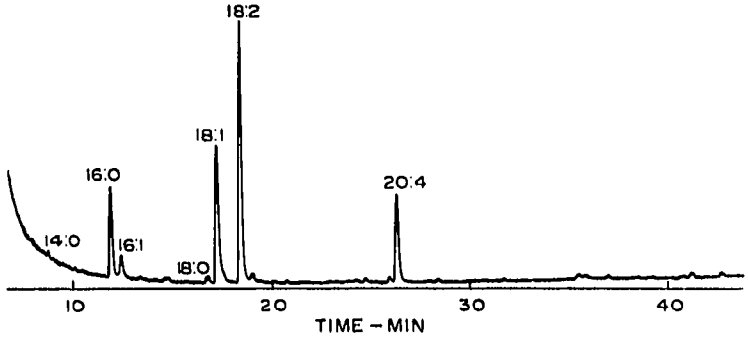

Fig. 4. GC analysis of the long-chain methyl esters from plasma cholesteryl esters of a stroke patient. Conditions, same as for Fig. 1. The acids are the same as in Fig. 3. A marked decrease in the linoleic acid to oleic acid ratio is evident. Data for all stroke patients are in Table II.

TABLE II

LONG-CHAIN ACID COMPOSITION OF PLASMA CHOLESTERYL ESTERS OBSERVED FOR NORMAL SUBJECTS AND FOR STROKE PATIENTS

Acid						
16:0	*16:1*	*18:0*	*18:1*	*18:2*	*20:3**	*20:4*
*Normal subjects***						
17.9	2.1	2.7	19.5	53.1	—***	4.6
11.7	3.1	2.3	23.1	55.4	—	4.4
16.8	3.7	2.8	22.5	50.3	—	4.1
15.9	1.9	2.3	21.6	54.5	—	3.9
11.4	4.1	2.3	18.7	57.8	0.6	5.1
17.6	0.9	1.8	21.8	49.5	0.3	7.8
10.7	2.3	1.7	18.6	57.2	0.4	8.7
8.2	—	—	12.6	74.1	—	4.8
10.1	2.7	0.6	20.6	63.2	—	2.6
6.9	—	2.1	17.3	66.0	—	7.3
10.2	1.0	1.0	18.2	65.3	—	4.0
12.5	3.1	1.1	20.1	58.0	—	4.8
11.2	3.5	0.6	18.6	60.9	—	4.8
*Stroke patients***						
18.8	6.3	3.1	21.9	39.6	—	10.4
8.7	3.8	3.1	23.9	44.4	—	16.1
11.6	2.9	5.3	24.5	40.5	—	15.1
10.9	3.1	1.1	23.1	44.6	—	17.2
11.9	2.0	0.9	23.0	48.3	0.5	13.4
9.6	3.5	1.4	26.8	48.3	0.6	9.8
15.7	2.7	2.2	20.7	48.9	0.4	8.9
12.6	5.5	0.8	31.2	39.6	0.7	9.2
12.3	5.1	0.7	27.1	47.9	—	7.4
10.9	5.2	0.7	31.3	45.2	—	6.3
11.8	2.4	1.3	31.5	44.4	0.4	7.7
9.4	2.9	0.9	31.3	47.2	0.3	7.7
10.0	3.8	0.6	22.2	57.1	—	6.1
13.4	2.6	1.4	20.8	53.1	—	8.4
10.1	1.7	0.6	24.9	52.1	0.2	10.7
12.5	3.3	1.4	22.2	45.9	—	14.4
11.0	—	1.1	20.6	62.1	—	4.9
14.3	2.6	1.5	22.0	51.9	—	7.4
9.2	3.1	0.9	24.6	54.7	0.2	6.9

* This acid is 20:3ω6, according to earlier studies.

** These data are in accordance with published values. Goodman and Shiratori[21] carried out analyses for two normal subjects and for a patient with atherosclerotic heart disease and mild diabetes mellitus, one year after myocardial infarction. For the normal subjects, the oleic, linoleic and arachidonic values observed were 24.6, 50.9 and 7.7% (ratio: 2.07), and 21.4, 56.0 and 5.8% (ratio: 2.61), respectively. The patient values were 24.5, 45.4 and 7.7% (ratio: 1.85) (falling in the patient category according to Table III), respectively. The early study by Hanahan *et al.*[22] lacks a value for arachidonic acid, although this is a known constituent of human plasma cholesteryl esters. The oleic acid and linoleic acid values were 22 and 51%, respectively.

*** Below 0.2%.

TABLE III

PLASMA CONCENTRATION OF α-TOCOPHEROL, AND RATIOS OF LINOLEIC ACID TO OLEIC ACID IN PHOSPHATIDYLCHOLINES AND IN CHOLESTERYL ESTERS OBSERVED FOR NORMAL SUBJECTS AND FOR STROKE PATIENTS

PC = Phosphatidylcholine; CE = cholesteryl ester.

*Normal subjects**			*Stroke patients**		
α-Toc (mg/dl)	*Lin/Ole ratio*		*α-Toc (mg/dl)*	*Lin/Ole ratio*	
	PC	*CE*		*PC*	*CE*
0.70	2.73	2.72	0.12	1.67	1.81
0.51	2.16	2.40	0.20	1.37	1.86
1.12	1.95	2.24	—**	1.14	1.65
1.07	2.21	2.53	0.18	1.51	1.93
1.20			0.18	1.48	2.09
0.62	3.14	2.82	0.18	1.80	1.80
0.71	3.44	2.27	0.15	1.62	2.36
0.75	2.63	3.08	0.31	1.34	1.27
0.58	3.47	5.88	—	1.00	1.77
0.50	2.89	3.07	0.26	0.72	1.44
1.22	2.32	3.82	—	1.18	1.41
0.51	1.97	3.59	0.18	1.38	1.51
0.95	2.62	2.90	0.58	1.68	2.57
	1.95	3.27	0.57	1.77	2.55
			0.24	1.31	2.09
			0.28	1.13	2.07
			0.20	1.43	3.04
			0.11	1.50	2.36
			0.25	1.80	2.22

* The order is the same as in Tables I and II.
** Below 0.1%.

α-Tocopherol concentrations (Table III) were determined by GC analysis as described previously[1].

Relationship between plasma phosphatidylcholine composition and α-tocopherol concentration

Values for linoleic acid to oleic acid ratios and α-tocopherol concentrations for stroke patients and normal subjects are shown in graphic form in Fig. 5. There is no reason to doubt the validity of these analytical results, but because of the potential significance of the boundary relationship evident in Fig. 5 it is desirable to examine other evidence linking α-tocopherol depletion (observed with stroke patients) with alterations in phospholipid composition. The analytical data of Underwood *et al.*[6] show a significant decrease in linoleic acid content for patients with cystic fibrosis when compared with normal subjects, for all tissues that were examined. The average α-tocopherol concentration in plasma, for two groups of patients, was about 0.2 mg/dl. Compositional data for plasma phosphatidylcholines for cystic fibrosis patients, and for normal subjects, were reported recently by Rosenlund *et al.*[5]. The linoleic acid to oleic acid ratio for plasma phosphatidylcholines of normal children was 0.93; the same ratio for patients was 0.31 (values calculated from the averages given by Rosenlund *et al.*[5]).

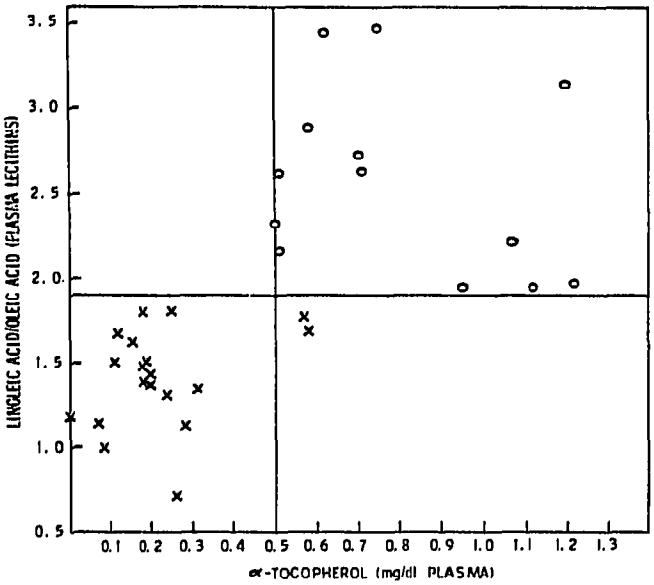

Fig. 5. Relationship between linoleic acid to oleic acid ratio in plasma phosphatidylcholines and plasma α-tocopherol concentration, for stroke patients (×) and normal subjects (○). These stroke patients are not the same individuals as those participating in a previous study[1]. With one exception (0.7 mg/dl), all stroke patients studied up to the present time have had plasma α-tocopherol concentrations below 0.6 mg/dl; the accepted limit below which erythrocyte structure is altered (susceptibility to peroxide-induced hemolysis) is 0.5 mg/dl[23].

The biological significance of our results is best discussed elsewhere, but several comments relating to the analytical aspects of this finding should be mentioned. The composition of any phosphatidylcholine depends both on enzyme kinetics for the incorporation of a specific long-chain acid, and upon substrate availability. Present evidence suggests that a generalized deficiency in linoleic acid exists for children with cystic fibrosis; this is probably due to inadequate absorption of dietary linoleic acid. The consequences are analytically detectable in several ways. The most pronounced change is in the long-chain acid composition of plasma phosphatidylcholines. For comparison purposes, this can be expressed in terms of an altered linoleic acid to oleic acid ratio. The compositional change for plasma cholesteryl esters is less marked when averages are compared. Very little change in composition was observed[5] for plasma triglycerides. The compositional changes seen in our work for linoleic acid in plasma phosphatidylcholines and cholesteryl esters are of exactly the same character as those seen in cystic fibrosis, and we are assuming at this time that the compositional changes are due to lack of substrate availability and not to altered enzyme kinetics for the incorporation of linoleic acid in phosphatidylcholines. If this view is correct, α-tocopherol depletion in adults leads to linoleic acid depletion by an undefined (and possibly indirect) mechanism. This depletion apparently occurs even when there is no difficulty in the absorption of linoleic acid.

The most likely route of linoleic acid loss is by normal β-oxidation. α-Tocopherol may protect the acid from oxidation, possibly through molecular complex

formation as envisaged by Lucy[9]. It is also possible, however, that the observed compositional effect is due to altered enzyme kinetics, and that α-tocopherol is a regulator governing the extent of utilization of linoleic acid in phosphatidylcholine synthesis. Further work is needed to establish the mechanism of the effect.

In either case, the size of the exchangeable pool of linoleic acid is reduced in the α-tocopherol-depleted state. Analytical measurement of this effect would not, however, define the mechanism. The chief reason for restrained judgment at this time with respect to mechanism lies in the results of the fish feeding experiments of Watanabe *et al.*[10] discussed briefly in the previous paper[1]. Their results indicate that the effect of α-tocopherol depletion is non-specific, suggesting that another substance is involved. If this is true, the analytical requirements for further study are quite different. For example, no methods exist at this time for the microdetermination of retinoic acid in tissue, although it seems a virtual certainty that this compound would be depleted at the same time that α-tocopherol is depleted, and it is known that retinoic acid is needed to sustain life. It is not impossible that an as yet unrecognized compound is involved in the mechanism(s) responsible for the observed associative effect.

Relationship between plasma cholesteryl ester composition and α-tocopherol concentration

The distribution of long-chain acids in cholesteryl esters is related to the structure of phosphatidylcholines because of the lecithin:cholesterol acyltransferase reaction. The compositional data shown in Table I indicate that the major change in structure for plasma phosphatidylcholines in the α-tocopherol-depleted state is replacement of linoleic acid by oleic acid. If this change is largely confined to the α-

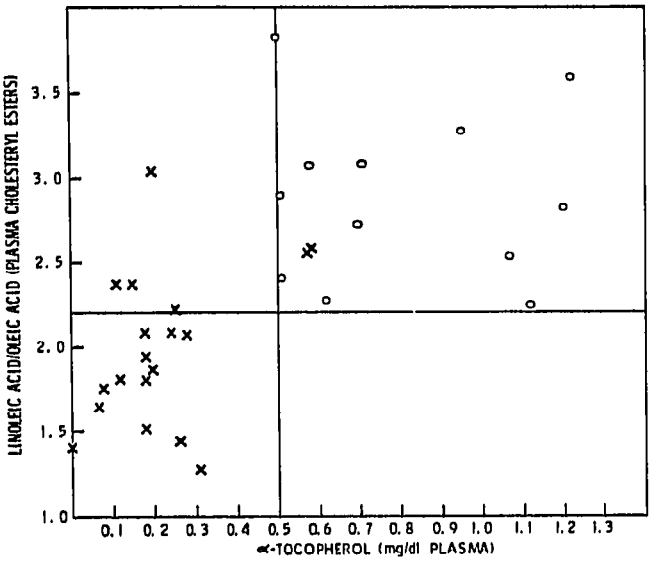

Fig. 6. Relationship between linoleic acid to oleic acid ratios in plasma cholesteryl esters and plasma α-tocopherol concentration, for stroke patients (×) and normal subjects (○). The results suggest that structural changes occur first in the α-position of phosphatidylcholines, but that the β-position may be affected as the availability (or degree of utilization) of linoleic acid decreases.

position, little or no change in cholesteryl ester composition would be expected. If the acyl group changes occur chiefly in the β-position, the linoleic acid to oleic acid ratio would be expected to change in parallel for both cholesteryl esters and phosphatidylcholines. Fig. 6 shows that some patients have ratios for cholesteryl esters which are not different from normal ratios, but that for many patients the ratio is depressed. This suggests that a temporal relationship may be involved (Binder *et al.*[11], in studies of α-tocopherol deficiency caused by lipid malabsorption, pointed out that progressive changes seem to be associated with a prolonged deficiency state). The successive stages may be: (1) α-tocopherol depletion, (2) linoleic acid depletion, (3) progressive structural changes in newly synthesized phosphatidylcholines which first affect the α-position, then the β-position, (4) progressive structural changes in cholesteryl esters involving the substitution of oleic acid for linoleic acid.

From an analytical point of view, there are no difficulties in compositional analysis of either phosphatidylcholines or cholesteryl esters, except for the lack of quantification with respect to bond and *cis/trans* isomers of oleic acid. There is only slight evidence of the presence of isomers in plasma cholesteryl esters (Figs. 3 and 4), suggesting that the isomers evident in Figs. 1 and 2 are largely present as acyl groups in the α-position. The biological effects of changes of this kind in phosphatidylcholine structure are unknown.

Relationship of arachidonic acid content of phosphatidylcholines to α-tocopherol concentration and to linoleic acid to oleic acid ratios

Compositional analyses of plasma phosphatidylcholines of patients showed an increase in arachidonic acid content in some instances. This effect is shown in Figs. 7 and 8, in which the arachidonic acid content is shown in relation to plasma α-

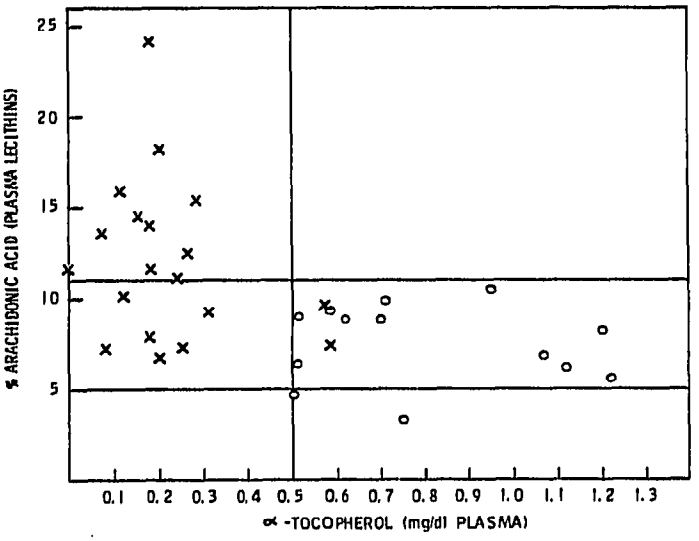

Fig. 7. Relationship between arachidonic acid content of plasma phosphatidylcholines and plasma α-tocopherol concentration, for stroke patients (×) and normal subjects (○). The results suggest that arachidonic acid is increasingly available as a substrate for phosphatidylcholine synthesis for some patients.

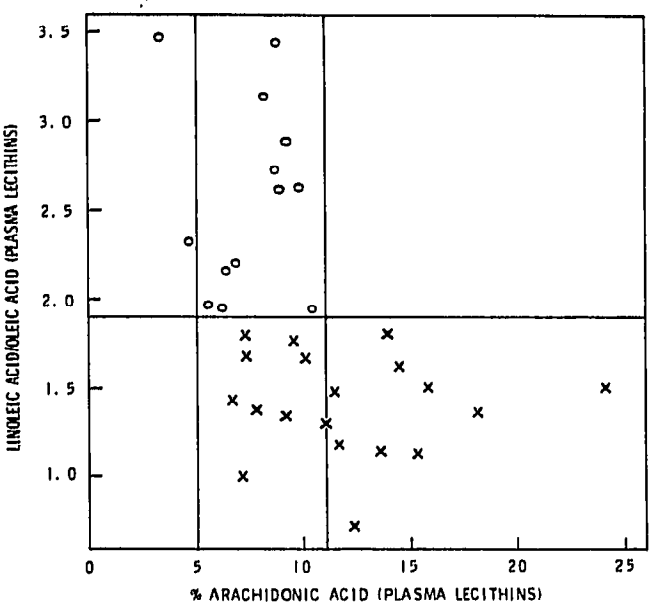

Fig. 8. Relationship between linoleic acid to oleic acid ratio in plasma phosphatidylcholines and arachidonic acid content in plasma phosphatidylcholines, for stroke patients (×) and normal subjects (○). The transition or boundary between the patient group and the normal group occurs when the linoleic acid to oleic acid ratio in phosphatidylcholines is about 1.8–2.0, and when (Table III) the plasma α-tocopherol concentration falls to 0.5–0.6 mg/dl. In a few instances in other studies we have observed an arachidonic acid content in normal plasma phosphatidylcholines of 3–5%. Most young normal subjects show 5–11% of arachidonic acid: the average value reported[5] for normal children was 7.9%. It is not known if the exchangeable or utilizable pool of arachidonic acid is primarily derived from dietary sources or from biosynthesis.

tocopherol concentration and to the linoleic acid to oleic acid ratio values. Fig. 8 suggests that a fifth stage should be added to the proposed sequence of changes: (5) increased incorporation of arachidonic acid may follow a decrease in incorporation of linoleic acid. This effect presumably requires increased substrate availability. It is not known if the increased supply of arachidonic acid is from dietary sources or is a result of increased biosynthesis.

These results are not open to immediate interpretation in mechanistic terms, but they agree with other recent studies. Platelet aggregation is believed to be due to enzymic formation of the endoperoxides described by Samuelsson and coworkers[12–14] and by others[15–17]; arachidonic acid is the required substrate. The later stages leading to stroke may be: (6) increased availability of arachidonic acid from dietary sources or by biosynthesis, and (7) use of arachidonic acid by platelet enzymes to form endoperoxides.

Risk analyses

Plasma concentrations of α-tocopherol below the 0.5–0.6 mg/dl boundary may indicate risk. If the linoleic acid to oleic acid ratio found for plasma phosphatidylcholines falls below 1.8–2.0, risk may be increased. An elevation in arachidonic acid

content of plasma phosphatidylcholines may reflect a condition of high biochemical risk, because of an increased supply of arachidonic acid.

The data of Rosenlund *et al.*[5], if interpreted in this way, would indicate that children with cystic fibrosis are at risk with respect to stroke. However, no evidence of increased availability of arachidonic acid was reflected by their compositional data. The arachidonic acid content of plasma phosphatidylcholines was lower for the patient group (4.9%) than for normal children (7.9%). This finding suggests that increased availability of arachidonic acid may be a basic requirement for the occurrence of stroke, and that the risk associated with α-tocopherol and linoleic acid depletion is much less significant than the risk associated with increased availability of this acid. Adults with an adequate dietary supply of linoleic acid may not show an increase in arachidonic acid availability, however, if α-tocopherol is not depleted. A condition of minimum risk may well involve an α-tocopherol concentration of 1.0 mg/dl or more, normal substrate availability of linoleic acid, and low substrate concentration of arachidonic acid. These conditions can be recognized through the use of the analytical procedures described in this and the previous paper[1].

CONCLUSION

Analyses of plasma lipids can be carried out by the methods described in this and the previous paper[1]. Plasma concentrations of α-tocopherol, linoleic acid to oleic acid ratios in free fatty acids, phosphatidylcholines and cholesteryl esters, and relative amounts of arachidonic acid in phosphatidylcholines and cholesteryl esters, were determined for normal young adult subjects and for stroke patients. The results indicate the existence of altered lipid metabolism and altered phosphatidylcholine structures in stroke patients. The observed effects may have their origin in slow depletion of α-tocopherol and linoleic acid due to differences in rates of metabolism and rates of absorption. The precise mechanism(s) involved in the metabolic changes are unknown. It may be possible to define conditions of minimum biochemical risk and high risk of thrombotic disorders by use of these lipid analytical procedures.

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