The lipid composition of human plasma chylomicrons

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SUMMARY The lipid composition of human chylomicrons has been studied using blood collected from normal male volunteers after ingestion of 200 g of butter fat. The concentration of chylomicrons in the blood samples varied widely among 12 subjects but in each case the following lipids were present: triglycerides, diglycerides (probably the 1,2-isomers), monoglycerides, free and esterified cholesterol, traces of free fatty acids, phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin (two components), and lysolecithin. The fatty acid composition of chylomicron triglycerides was usually similar to that of butter, but myristic and shorter-chain acids showed concentrations lower than those in butter.

Pooled phospholipids were fractionated by preparative thin-layer chromatography. Phosphorus determinations indicated the following proportions of components (mole per cent of total phospholipids): phosphatidyl ethanolamine region, 6%; phosphatidyl choline, 79%; sphingomyelins, 12%; lysolecithin, 4%. The fatty acid compositions of the chylomicron phospholipids have been determined. The total sphingomyelin region contained C_{22}, C_{23}, C_{24}, and C_{24} monoenoic acids as major components.

Classification and identification of the various hyperlipidemias is a clinical problem of some urgency. It seems possible that unusual distribution of lipids within the various lipoprotein density classes may be present in these disorders, in addition to the abnormally high levels of one or more lipids in the plasma as a whole.

A better understanding of the normal particle must be gained before any abnormalities can be defined in the chylomicron of the hyperlipidemic subject. The lipid composition of human plasma lipoproteins of density less than 1.019 has been studied in certain aspects by Phillips (2), while Nelson (3) has separated the phospholipids of serum lipoproteins (d < 1.20) using silicic acid column chromatography, and studied the constituent fatty acids. Recently Bierman et al. (4) have separated two types of chylomicrons from plasma during alimentary lipemia, using zone electrophoresis and differential flocculation with polyvinylpyrrolidone; "primary" particles were thought to originate in the intestine and "secondary" particles in the liver. The fatty acid composition of the total phospholipids and of the phosphatidyl choline associated with these two types of particle was recorded. Other information concerning the lipids of chylomicrons is collected in a recent review (5).

In the present study an attempt has been made to characterize chylomicrons obtained after feeding butter to normal men. Thin-layer chromatography (TLC) now provides an excellent method for fractionation of phospholipids, and modifications of the technique have been used for preparative purposes and also for monitoring the various procedures.

METHODS

An outline of the experimental procedure is shown in Fig. 1.

All solvents were Reagent Grade, distilled before use. An atmosphere of nitrogen was maintained over the extracted lipids whenever practicable. Exposure to air occurred while silicic acid bearing the adsorbed lipids was being scraped from the thin-layer plates.

Preparation of Washed Chylomicrons

Each of 12 normal men (aged 29–33 years; fasting plasma glycerides less than 130 mg/100 ml) was given a liquid

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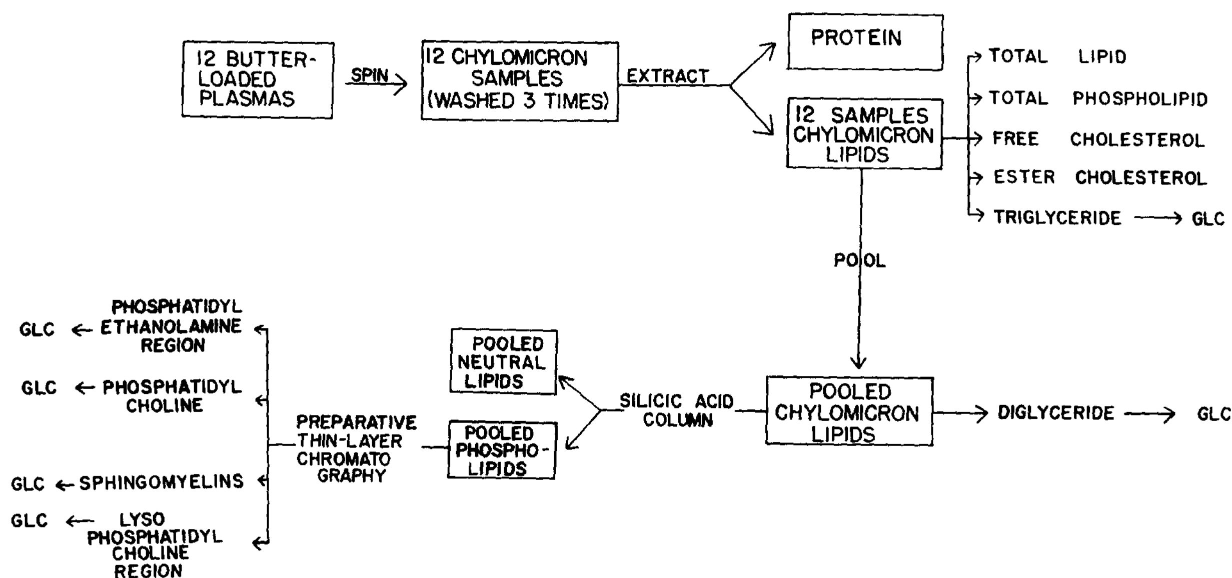

FIG. 1. Outline of procedure.

formula by mouth, containing 200 g of butter fat. About 8 hr later 160 ml of venous blood was drawn from each subject into EDTA tubes and spun at 2° to obtain plasma. Turbidity varied from slight to heavy. Sixty milliliters of plasma from each subject was processed using a Spinco Model L preparative ultracentrifuge with a 40.3 rotor. Plasma was mixed with saline of density 1.006 g/ml and spun at 20,000 rpm for 30 min (8.6×10^5 *g*-min). The upper chylomicron-containing layer was separated and washed by mixing with saline (1.006 g/ml) and spinning at 20,000 rpm for 60 min (1.7×10^6 *g*-min). Chylomicrons were separated and the washing procedure was repeated twice more to give 12 samples of "washed chylomicrons."

The freedom of chylomicron preparations from more dense lipoproteins is difficult to establish. In some of our samples the weight, phosphorus content, and thin-layer chromatographic pattern of both washings and "washed chylomicrons" were studied after each of the three washing steps. It was noted that the weight of the material washed out of the chylomicrons decreased with consecutive washings, and that the percentage phosphorus content of the material washed out decreased and approached that of the final washed preparation. Thin-layer chromatograms (Fig. 2) also suggested that the material removed in the first washing contained more cholesterol and phospholipid (relative to triglyceride) than did the lipid removed by the third washing.

Extraction of Lipids

Total lipids were extracted from each chylomicron sample and washed according to the procedure of Folch et al. (6). Thin-layer chromatography of washing concentrate indicated that no loss of lysolecithin or other phosphos lipids occurred. The small quantities of chylomicron protein precipitated during extraction were filtered off on glass wool and determined by nitrogen estimation.

Thin-Layer Chromatography (TLC)

Glass plates (20 x 20 cm or 20 x 26 cm) were coated with a layer of Silica Gel G (E. Merck AG, Darmstadt, Germany) about 275 μ in thickness, and briefly dried at 110°. They were then "washed" by allowing a polar solvent mixture (Solvent II, see below) to rise to the top of the plates in closed tanks lined with filter paper. This procedure removed to the top of the plates impurities in the silica gel that otherwise interfere with gas–liquid chromatography (GLC) and phosphorus determination on separated lipids. The washed plates were activated at 110° for 1 hr before use.

Petroleum–diethyl ether–acetic acid 80:20:1 v/v/v (Solvent I) was used for separation of the less polar lipids; and chloroform–methanol–water 80:35:5 v/v/v (Solvent II) for separation of individual phospholipids. When plates were used only for visual checking of composition, spots of lipid solutions were applied to 20 x 20 cm plates and after development the entire area was sprayed with 50% (v/v) sulfuric acid and heated at 250° on a hot-plate to visualize the separated lipids.

In order to separate triglycerides and diglycerides from total chylomicron lipids for GLC, lipids (4 mg) were applied in chloroform as a streak to 20 x 20 cm plates which were developed in Solvent I. The plates were then

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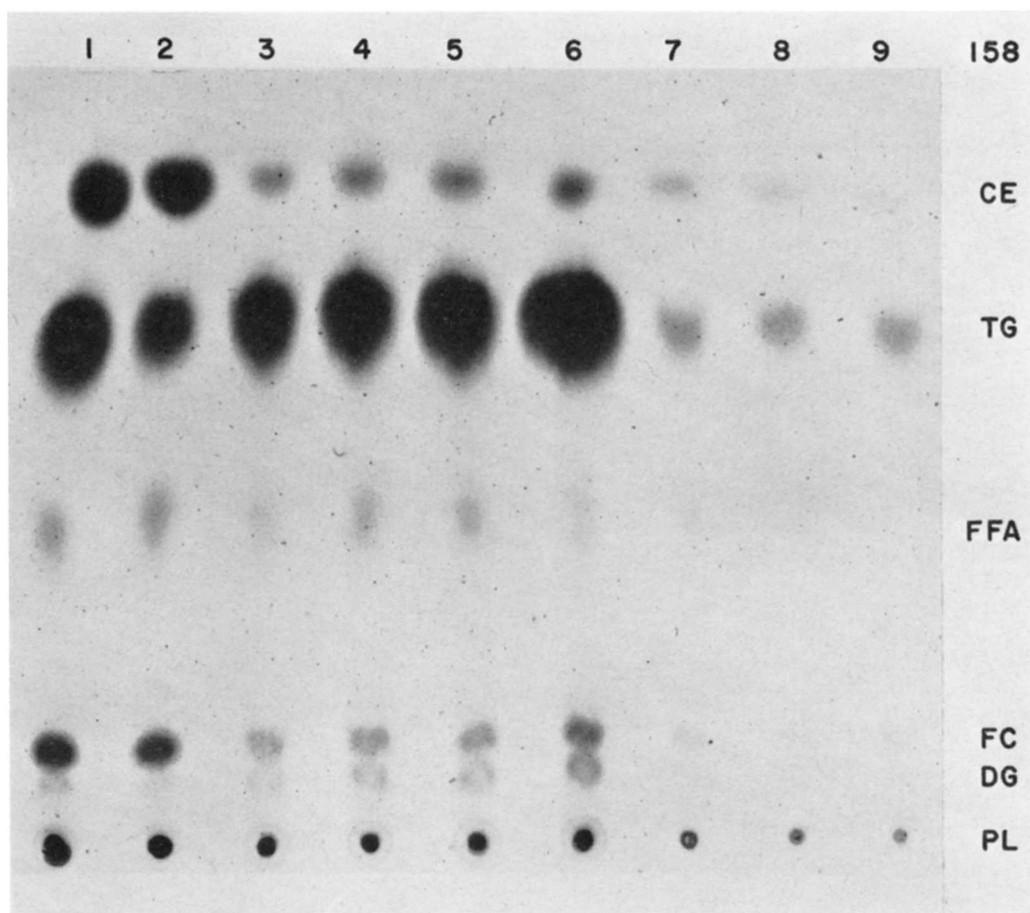

FIG. 2. Thin-layer chromatogram showing less polar lipids of a fat-loaded plasma. 1, total lipids; 2, infranatant sample from ultracentrifugal fractionation; 3, crude chylomicrons; 4, ditto, after the first wash; 5, ditto, after the second wash; 6, ditto, after the third wash; 7, lipids removed in the first wash; 8, lipids removed in second wash; 9, lipids removed in third wash. *CE*, cholesterol esters; *TG*, triglycerides; *FFA*, free fatty acids; *FC*, free cholesterol; *DG*, diglycerides (probably 1,2-isomers); *PL*, total phospholipids, at origin.

partially masked by clipping two glass plates over the surface of the silicic acid so that only two strips remained uncovered. Exposure to iodine vapor in a tank showed the position of the lipids in these strips, and on this basis the covered areas of silicic acid were scraped off so as to include only the particular lipids required. The success of the procedure could be checked by spraying the remainder of the plate with sulfuric acid (Fig. 3). After elution from the silicic acid in a small column with diethyl ether, sufficient triglyceride was obtained from one plate for GLC of the constituent acids, but it was necessary to pool the diglyceride region from four plates for this purpose. The removal of some of the adjacent free cholesterol band with the diglycerides did not interfere with the subsequent GLC.

Separation of phospholipid classes was achieved by applying 5 mg of total phospholipids in chloroform as a streak to longer plates (20 x 26 cm) and developing in Solvent II. A similar masking and visualization procedure was used to obtain four bands containing phosphatidyl ethanolamine and phospholipids of similar R_f, phosphatidyl choline, sphingomyelins, and lysolecithin. The silicic acid containing a separated phospholipid from a single plate was transferred to a small glass column and eluted with 10 ml of chloroform–methanol–water (80:35:5 v/v/v) followed by 10 ml of methanol. Evaporation of the combined eluate yielded the phospholipid.

Completeness of recovery of phospholipids from Silica Gel G has been investigated using total plasma lipids. At the loading levels used (16 μg total phospholipid phosphorus, or more, per plate) phosphorus recoveries ranged from 97 to 103 %. For good recoveries of phospholipids, the following points are important: (*a*) the lipid extract applied must be free of inorganic phosphorus and oxidized phospholipids; (*b*) removal of silicic acid

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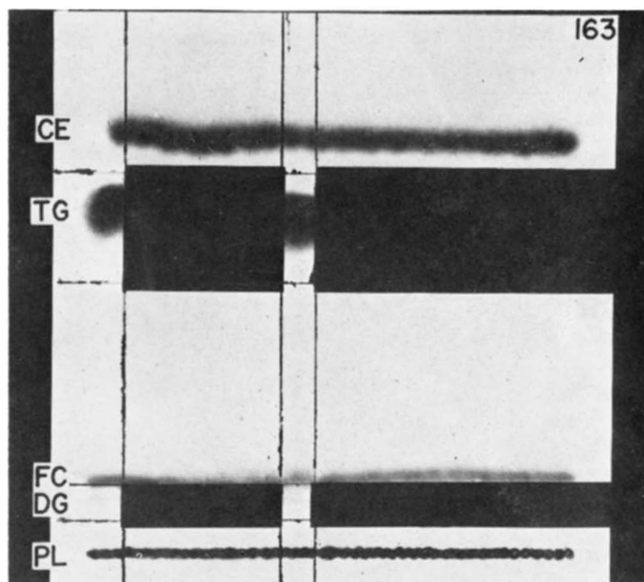

FIG. 3. Thin-layer chromatogram from which triglyceride and diglyceride regions have been scraped to provide samples for GLC of methyl esters. Success of procedure checked by spraying remainder of plate with sulfuric acid and charring. Key to lipid regions as in Fig. 2.

from plates, and elution, should be done as rapidly as possible; (*c*) exposure of the entire plate to iodine vapor must be avoided, as low recoveries of both phosphorus and unsaturated fatty acids result. With these conditions fulfilled, and using the eluting solvents described, we have found phospholipid recoveries to be satisfactory.

The separation obtained is illustrated in Fig. 4. The adequacy of separation was checked by TLC of the separated components. Separation of phosphatidyl choline and lysolecithin was excellent but the sphingomyelins contained a trace of phosphatidyl choline; some oxidative deterioration of the phosphatidyl ethanolamine region was apparent in spite of precautions taken to avoid this.

Column Chromatography

Neutral lipids and free fatty acids were separated from phospholipids on a column (1.5 x 10 cm) of silicic acid (Bio-Rad Laboratories, Richmond, Cal.) prepared in chloroform. About 650 mg of total lipids were applied to the column. Elution with chloroform removed neutral lipids and free fatty acids; it was followed by elution with chloroform–methanol 1:1 followed by pure methanol to remove phospholipids. Separation of the two fractions was complete, as judged by TLC.

Gas–Liquid Chromatography (GLC)

Triglycerides, diglycerides, and separated phospholipids were heated with 2% (v/v) sulfuric acid in methanol at 80° in sealed glass tubes for 1.5 hr; for sphingomyelins, 5% (v/v) sulfuric acid in methanol was substituted and heating was continued for 6 hr. The reaction mixture was diluted with water and the methyl esters were extracted with petroleum ether. Completeness of methanolysis was confirmed for each phospholipid class by running samples of the reaction products on thin-layer plates (Solvent II), when complete disappearance of the phospholipid spot was noted, with appearance of the methyl ester spot. The stability of unsaturated fatty acids under the conditions used for methylation of sphingomyelins was checked by GLC and found to be satisfactory.

Gas–liquid chromatography of methyl esters was carried out using a Barber-Colman Model 10 chromatograph under the following conditions: column, 25% diethylene glycol succinate polyester on Chromosorb W, 6-ft, 180°; detector, argon ionization (Sr^{90}), 500 v; argon flow rate, 50 ml/min; sample applied, about 100 μg. Acids were identified before and after catalytic hydrogenation of samples, by use of standards and in some cases graphically. Satisfactory linearity of detector response was demonstrated by checking with N.I.H. standard methyl ester mixtures.

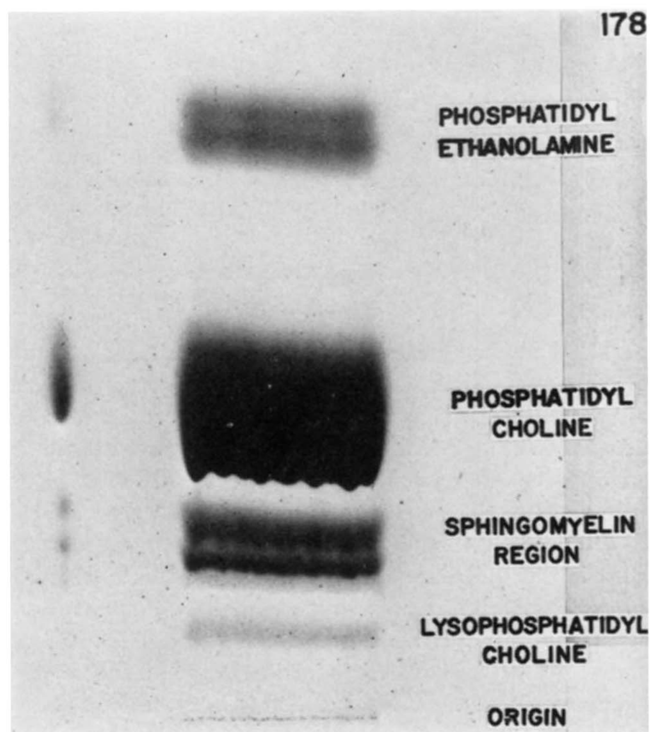

FIG. 4. Thin-layer chromatogram on long plate (20 × 26 cm) of phospholipids of pooled chylomicron lipids. The four major phospholipid classes are clearly separated; the sphingomyelin region contains two components; and the partial separation of two components within the phosphatidyl ethanolamine region is apparent. Left: single spot applied; right: streak applied.

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TABLE 1 Amounts of Chylomicron Lipids Contributed to Pooled Sample by Sera from 12 Subjects

Subject	Total Lipid	Phospholipid
	mg	*mg*
1	148	7.3
2	122	17
3	81	5.3
4	69	7.6
5	69	4.6
6	35	2.6
7	32	4.7
8	32	2.7
9	28	1.8
10	14	0.7
11	13	1.3
12	7	0.8

Analytical Methods

Total lipids in chylomicron extracts were determined by evaporating extracts to dryness and weighing.

Protein nitrogen was determined by the Kirk modification of the Kjeldahl method (7); free and total cholesterol (in duplicate) by the method of Michaels et al. (8); and lipid phosphorus (in duplicate) by a modification of the Fiske and Subarrow method described in reference 9.

RESULTS

The contribution made by the samples from each of the 12 men to the pooled chylomicron total lipids and phospholipids is shown in Table 1. Clearly there was a wide variation among the 12 subjects in the concentration of chylomicrons in the blood at the time of sampling; and the chylomicron phospholipid pool was dominated by the large contribution from three or four men.

The constituents in the total chylomicron lipids for each subject were studied by TLC in two solvent systems. An equal weight of lipid was applied for each sample. In the less polar solvent system (I) each sample showed a large triglyceride spot and, in addition, cholesterol esters, free fatty ac ds (traces), free cholesterol, diglycerides (probably 1,2-isomers) and unresolved phospholipids at the origin. In the more polar solvent system (II) traces of monoglycerides could be seen, and the following phospholipids: phosphatidyl ethanolamine region, phosphatidyl choline, sphingomyelins (two components) and lysolecithin.

Quantitative determination of some of the components of the chylomicron was carried out, and the mean values found, together with the range among 12 subjects, are shown in Table 2.

Constituent acids from chylomicron triglycerides were determined by GLC of their methyl esters. Results for four subjects showing relatively high concentrations of circulating chylomicrons are shown in Table 3, together with results for a composite diglyceride fraction. The fatty acid composition of the butter fed in this experiment, determined under the same conditions, is also shown.

TABLE 2 Composition of Chylomicrons*

Total Weight	Chylomicron Protein	Chylomicron Lipid: Phospholipid	Chylomicron Lipid: Free Cholesterol	Chylomicron Lipid: Ester Cholesterol
mg/100 ml plasma	%	%	%	%
117	2.5	9.0	0.8	1.7
(22–294)	(0.6–6.7)	(4.9–14.5)	(0.3–2.3)	(0.9–3.1)

* Mean of 12 samples, with range in parentheses.

After separation of constituent phospholipids by TLC, the relative proportion of each was determined by phosphorus estimation, with the results shown in Table 4.

Table 5 shows the fatty acid composition of each phospholipid class.

DISCUSSION

In order to obtain sufficient material for investigation of minor lipid components of chylomicrons, it was necessary to take a total of 2 liters of blood from 12 volunteers, following fat loading. The same volume of plasma was processed for each subject, but the contribution of total chylomicron lipids and phospholipids to the pool varied considerably (Table 1). The result was not unexpected in view of the known individual variation in degree of alimentary lipemia following ingestion of a fat load. Subdivision of the chylomicrons, for instance by further ultracentrifugation (10) or by zone electrophoresis (4), was not attempted in the present work.

The mean values for protein, phospholipid, and free and esterified cholesterol in chylomicrons of the 12

TABLE 3 Fatty Acid Composition of Chylomicron Triglycerides, Chylomicron Diglycerides and Butter (Mass Per Cent of Total Methyl Ester Sample)

	12:0	14:0	16:0	16:1	18:0	18:1	18:2
Triglycerides							
Subject 1	0.5	7.0	30.3	2.8	13.3	29.8	6.4
Subject 2	0.1	2.6	28.6	3.2	10.0	37.1	12.2
Subject 3	0.8	9.0	32.9	2.7	14.1	28.0	4.0
Subject 4	0.2	4.7	29.9	3.2	15.7	30.8	3.6
Diglycerides							
Composite from samples 1–4	0.1	4.1	28.0	4.3	12.4	30.8	6.7
Butter							
Used in fat loading	2.6	12.1	33.1	2.7	13.1	24.1	2.6

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TABLE 4 Distribution of Major Phospholipid Classes in Various Human Plasma Lipoprotein Fractions (Mole Per Cent of Total Phospholipids)

	Phosphatidyl Ethanol-amine Region	Phosphatidyl Choline	Sphingo-myelins	Lysolecithin Region
Chylomicron phospholipids (present study)	5.6	78.5	11.7	4.2
Plasma lipoprotein (d < 1.019, phospholipids. Phillips (2)	5.7	66.2	23.1	5.1
Serum lipoprotein (d < 1.20) phospholipids. Nelson (3)	5.6	71.2	20.2	3.0

subjects (Table 2) were similar to typical values found in previous studies (4, 5), although considerable individual variation was seen. The mean protein content of the 12 chylomicron samples (2.5%) was a little higher than the protein contents reported by some other workers for chylomicrons, but our value is influenced by contributions from several subjects with very low chylomicron levels (Table 1). The protein content of chylomicrons from subjects with these low levels tended to be higher than the mean, with a maximum of 6.7%, while the corresponding values for subjects with relatively high chylomicron levels were between 0.5 and 2%. Thin-layer chromatograms indicated that very minor amounts of free fatty acids were present in chylomicron lipids, but showed the presence of partial glycerides. A component on the chromatograms, moving slightly behind the cholesterol spot, was shown to contain combined fatty acids and was in the correct position for 1,2-diglycerides. Diglycerides did not result from hydrolysis during ultracentrifugal procedures since this component was visible on chromatograms of fresh total plasma lipids. After the initial separation of chylomicrons from denser lipoproteins, diglycerides were seen to accompany the chylomicrons and were almost absent from the infranatant fractions (Fig. 2). Small amounts of material moved to the correct position for monoglycerides. In each subject all four major phospholipids normally found in total plasma were present.

The fatty acid composition of chylomicron triglycerides (Table 3) showed a fairly close resemblance in some samples to the composition of the butter fed, but in others showed greater divergence. Presumably chylomicron preparations from some subjects contained large proportions of particles derived directly from the ingested fat, while those from other subjects probably contained significant proportions of particles in which the glyceride fatty acids were not directly derived from butter. Values for lauric and myristic acids were considerably lower than those found (by the same methylation technique) in the butter fed. This lends support to the concept that lower fatty acids in the diet, though absorbed and rapidly utilized, are not transported to a significant extent in the chylomicrons. The fatty acid composition of the composite diglyceride fraction was similar to that of the triglycerides.

The phospholipids of the chylomicrons were found by phosphorus analysis to consist largely of phosphatidyl choline with smaller amounts of sphingomyelin, phosphatidyl ethanolamine, and lysolecithin. It is clear from Table 4 that the distribution of these phospholipid fractions in our chylomicron preparation is quite similar to that found by Phillips (2) for lipoproteins of density less than 1.019 and to that found by Nelson (3) for lipoproteins of density less than 1.20. The lower proportion of sphingomyelin and higher proportion of phosphatidyl choline present in chylomicrons may reflect a more complete separation of these phospholipids by TLC than was possible by column chromatography. Alternatively, a low sphingomyelin content may be a true feature of chylomicron phospholipid make-up.

The fatty acid composition of the separated phospholipids of chylomicrons is shown in Table 5. The acids of the major phospholipid, phosphatidyl choline, are similar to those noted by Nelson (3) for lipoproteins of density less than 1.20 except that he found larger proportions of 20:4 and 22:6 acids. Bierman et al. (4) studied the fatty acids of phosphatidyl choline in both primary and secondary chylomicrons following butter or corn oil feeding, and found the pattern to be considerably in-

TABLE 5 Major Fatty Acids of Chylomicron Phospholipids (Mass Per Cent of Total Methyl Esters)*

	16:0	16:1	18:0	18:1	18:2	20:0	20:3	20:4	22:0	22:6	23:0	24:0	24:1
Phosphatidyl choline	34	1	16	13	25	0	2	5	0	1	0	0	0
Sphingomyelins	28	2	10	4	2	4	0	0	13	0	8	8	16
Phosphatidyl ethanolamine	13	3	24	19	15	0	1	7	0	2	0	0	0
Lysolecithin	23	4	37	14	7	0	1	8	0	0	0	0	0

* Values quoted to nearest whole number.

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fluenced by the nature of the fat used in loading. The proportions of 18:2, 20:4, and 22:6 acids in lecithin following butter loading were considerably less for either primary or secondary particles than those found by us for total chylomicron lecithin. The presence of 25% of linoleic acid in our lecithin suggested that the fatty acid spectrum of this major chylomicron phospholipid is not greatly modified during butter loading.

The acids found for phosphatidyl ethanolamine are included for completeness, although, as noted, TLC suggested that some oxidative loss of more unsaturated acids may have occurred. Certainly the contents of 20:4 and 22:6 acids were considerably less than those found by Nelson for this phospholipid in serum lipoproteins (d < 1.20); but our linoleic acid content was double that found by Nelson, while the palmitic acid values were very close.

Large amounts of 20:0, 22:0, 23:0, 24:0, and 24:1 acids were found in the present study in sphingomyelins from chylomicrons. There was general similarity between the composition of the fatty acids and the composition of the fatty acids found by Böttcher and Van Gent for sphingomyelin in the atherosclerotic aortic wall (11) and by Sweeley in plasma (12), although Nelson (3) found lower amounts of the C_{20}-C_{24} acids and higher amounts of linoleic acid in the sphingomyelin from plasma lipoproteins. The partial separation of the sphingomyelin region into two bands during TLC (Fig. 4) has been noted also with total plasma lipids. It was found that the lower (more polar) sphingomyelin band contained a large proportion of palmitic acid, while the upper (less polar) band contained a large proportion of the long-chain acids (C_{20} and above) present in the total sphingomyelin region.

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