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THE CHEMICAL, PHYSICAL, AND BIOLOGICAL PROPERTIES OF HUMAN SERUM PHOSPHOLIPIDES

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Gary Joe Nelson
(Thesis)

January 1960

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ABSTRACT

The phospholipide compositions of human serum lipoproteins have been determined on ultracentrifugally separated serum lipoprotein classes by silicic acid column chromatography and infrared spectrophotometry. The lipoproteins were separated into three classes: S_f 20–400, S_f 0–20, and HDL-2, 3. The phospholipides were separated into a fraction containing phosphatidyl-ethanolamine and phosphatidyl serine, a lecithin fraction, and a sphingomyelin fraction. All phospholipides are present in each lipoprotein class, but their distributions in each class are sufficiently different to be characteristic. Analyses were run on sera from five individuals, and only small deviations from the average values were observed.

In a few cases the fatty acids of the separated phospholipides were analyzed by gas chromatography.

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INTRODUCTION

This study is concerned with the distribution and composition of the major phospholipides in normal human serum, particularly in respect to their occurrence as constituents of the various lipoprotein classes that serve as the carriers of lipides in blood. From the sera of each of five healthy persons, three distinct lipoprotein classes have been isolated, and their phospholipide compositions determined analytically by a combination of chromatographic, infrared spectrophotometric, and chemical methods. The interpretation of these data is discussed with respect to theories of lipoprotein transformations. Finally a unified theory of lipoprotein formation and fat transport is presented.

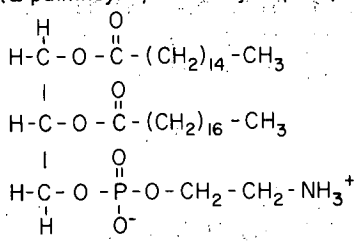
I

Studies on the phospholipides progressed rather slowly from the time of their initial discovery, credited to Fourcroy in 1793.[1] Vauquelin, in 1812, was the first to prove unequivocally that phosphorus must be part of the fatty material of the brain.[2] Later, in 1846, Gobley showed that egg yolk contained a phospholipide which he called lecithin, from λεκιθος, the Greek word for egg yolk.[3] In 1874 Thudichum proposed the generic term "phosphatide" for all compounds containing phosphorus that are generally lipide in their behavior.[4] More commonly the term "phospholipide," also commonly spelled "phospholipid," is used in technical literature today, and is used throughout this paper. (In this thesis the following abbreviations have been used: PE, phosphatidyl ethanolamine; PS, phosphatidyl serine (frequently these two compounds, PE and PS, will be abbreviated together as phosphatidyl ethanolamine-serine, PE-S); L, lecithin; S, sphingomyelin; PL, phospholipide; TLE, total lipide extract; CS, cholesterol; CSE, cholesterol ester; TG, triglyceride; FA, fatty acid; UFA, unesterified fatty acid; LDL, low-density lipoprotein; HDL, high-density lipoprotein.)

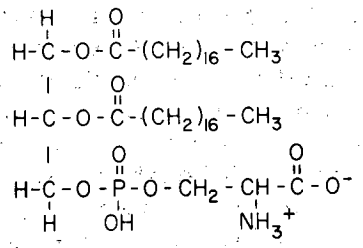
It has been only in the last few years that the structures of many of the phospholipides have been accurately determined.[5-9] Indeed, the structures of some remain in doubt, such as inositol phosphatides,[10] ganglioside,[11] etc. Figure 1 gives the structure of the principal phospholipides of human serum. In the older work the compounds phosphatidyl ethanolamine and phosphatidyl serine were grouped together, because of their similar chemical and physical properties, as "cephalin."

PHOSPHOLIPIDE STRUCTURE

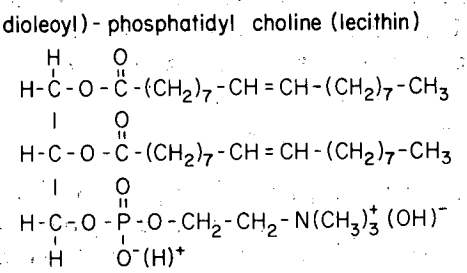
L - α - (α' palmitoyl - β - stearoyl) - phosphatidyl ethanolamine



L - α - (distearoyl) - phosphatidyl L - serine



L - α - (dioleoyl) - phosphatidyl choline (lecithin)



sphingomyelin (palmitic form)

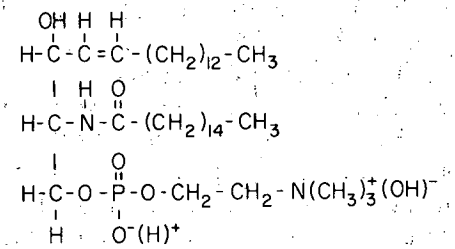


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Fig. 1. The chemical structures of the primary phospholipides of human sera. Phosphatidyl ethanolamine and phosphatidyl serine are usually analyzed as a single fraction. The fatty acid compositions shown in this figure are arbitrary but are believed to be representative of those found in serum.

This term has since been discarded in favor of the more specific chemical nomenclature. However, the two compounds are still frequently analyzed as a single entity, as in the studies of serum phospholipides; they are so treated in this work also.

Progress was slow in the study of phospholipides, primarily because of the difficulties that arose in separations and attempts to achieve pure samples. Handling procedures were crude and inadequate. As is true for all fats, there is rarely a unique chemical structure for a phospholipide. Thus, for a lecithin molecule, there can be several combinations of fatty acids, each causing changes in the physical characteristic of the molecule.

Although phospholipides had long been known to occur in human serum[12, 13, 14] and determinations of total serum phosphorus had been reported, no separations and determinations of the separate serum phospholipides were reported until the work of Kirk in 1938.[15, 16] He reported values for the concentrations of the major phospholipides in human serum and red blood cells. Kirk's procedures did not, in view of more modern knowledge, yield accurate results. There were several more analyses reported[17, 18, 19] soon after Kirk, none of which can be regarded as any more accurate.

The first reliable values for serum were reported by Hack in 1947,[20] and are, in view of recent work, correct within the variations for different individuals. Hack's report remained unconfirmed, however, until Phillips published results in good agreement with Hack's in 1958 (see presentation of data).[21]

A new development appeared in the methodology of lipids investigations with the introduction of chromatographic procedures for the separation of lipides by Trappe in 1940.[22] Some time later Borgström observed that phospholipides could be quantitatively separated from the non-phosphorus-containing lipides by use of silicic acid column chromatography.[23] Since then the literature has abounded with reports of chromatographic procedures for the study and separation of lipides, and phospholipides in particular. Lea, Rhodes, and Stoll were the first to report a fractionation of the phospholipides on silicic acid, utilizing egg yolk lipides.[24] Later Hanahan reported a method for tissue phospholipides,[25] while Marinetti et al. have reported chromatographic studies, using paper impregnated with silicic acid, that qualitatively separate almost every phospholipide.[26] Recently, methods of extended lipide fractionation have been reported by Freeman et al.,[27] Hirsch and Ahrens,[28] and Klein and Jansson[29] using silicic acid columns and changing solvents frequently. Phillips[21] and this author[30] have published silicic acid column chromatographic procedures specifically designed for separation of the phospholipides of human serum.

It has been generally assumed that the major phospholipides of serum are phosphatidyl ethanolamine, lecithin, and sphingomyelin. However, Phillips recently reported the isolation and determination of 7% lysolecithin in human serum.[31] No confirmation of this finding has

since appeared. The minor components are plasmalogens, inositol phosphatides, and phosphatidyl serine. The total of these minor compounds is not believed to exceed 1 or 2% of the total phospholipides.[32]

Previous studies on human serum phospholipides have dealt with concentrations in whole serum. With the introduction of the concept of lipoprotein classes (see below) it became of interest to study the phospholipides of serum as structural units of these macromolecules. Although there is now agreement on the relative proportions of the phospholipide species in whole serum, it has not previously been known how the individual phospholipides are distributed in the lipoprotein classes. While this work was in progress, Phillips reported such a study.[33] His results are discussed in some detail in the appropriate section. It was hoped that information on the phospholipide composition of the lipoprotein classes would bear on the larger problem of lipoprotein structure and lipide transport.

In the course of this study a gas chromatography unit became available in the laboratory. A corollary study of the fatty acid complement of the phospholipides was started in order to see if there was any relationship between fatty acid composition and lipoprotein class in the phospholipides. The almost complete lack of knowledge of the composition and distribution of serum phospholipide fatty acids was an incentive to characterize this facet of phospholipide structure.

Studies on the fatty acid composition of phospholipides have been primarily restricted to samples from tissues. Recently, Luddy et al. reported values for whole serum phospholipides as a single class, using alkali isomerization techniques.[34] They studied the pooled serum from two individuals and found 40% saturated fatty acids and 60% unsaturated. The unsaturated acids primarily oleic (35%), linoleic (15%), and arachidonic (8%), with traces of other unsaturated acids on the order of 1%, which is the limit of error of their method. The saturated group was not characterized further.

James and Lovelock also reported fatty acid analyses on total serum phospholipide fractions by gas chromatography.[35] They found a range of values for sera from ten subjects. The average values were as follows: palmitic, 30%, palmitoleic 2%, stearic 20%, oleic 20%, linoleic 20%, and arachidonic 8%. Mukherjee et al. reported the total phospholipide fraction fatty acid composition of rat serum using alkali isomerization.[36] They found rat serum phospholipides to be highly saturated, approximately 75%. In spite of these few instances, our knowledge of fatty acids of human serum phospholipides is very incomplete. And no data are available on the fatty acid composition of the individual phospholipides of human serum, or of the fatty acid composition of the phospholipides of the lipoprotein classes.

Before the introduction of gas chromatography by James and Martin in 1950,[37] fatty acid analysis was one of the most difficult operations in analytical chemistry. There was simply no adequate micro-method for the quantitative and qualitative identification of fatty acids. Now, with the technique of gas chromatography, as refined by Lovelock,[38] Lipsky,[39,40]

and Golay,[41] the possibility exists of separating and identifying 0.1 μg of a complex mixture of fatty acid methyl esters, with an ultimate resolving power of 10^9 molecules or better.

II

There can be no doubt that all lipides present in serum, except for a few unesterified fatty acids, exist in complexes of lipide and protein, generally termed lipoproteins. The first lipoprotein recognized as such was that from egg yolk, described in 1867 by Hoppe-Seyler.[42] It is now generally accepted that all lipides in cells are bound in lipoproteins, with the possible exception of adipose tissue. It nevertheless remains true that there has been little success in isolating pure lipoproteins from tissue.

Macheboeuf is credited with being the first to isolate a lipoprotein from serum,[43] but his material was grossly contaminated by other serum proteins. Early methods of obtaining serum lipoproteins involved precipitation by increasing the ionic strength of the medium, or precipitation with alcohol[44,45] or other solvents at low temperature. These procedures invariably resulted in impure samples. Similar attempts at purification by electrophoresis[46] also failed to yield pure material.

Soon after the introduction of the ultracentrifuge by Svedberg,[47] attempts were made to isolate lipoproteins from serum with this tool. When whole undiluted serum was ultracentrifuged, certain anomalies were immediately apparent. These phenomena, resulting from the Johnston-Ogston effect, delayed the application of the ultracentrifuge to isolation of serum lipoproteins. The anomaly in the observed schlieren pattern, termed the "X" protein by McFarlane[48] and Pederson,[49] was finally explained by Gofman et al. in 1949,[50] utilizing the theory of Johnston and Ogston[51] which treated the problem of a mixture of proteins of different densities undergoing sedimentation in an ultracentrifuge. Gofman et al.[50] recognized that flotation, rather than sedimentation, was occurring, and that the density of some lipoproteins was less than that of serum. The ultracentrifugal flotation procedure yielded the first purified lipoprotein from human serum.

In a series of papers, Gofman and associates (50, 52, 53, 54) showed that human serum contained a spectrum of lipoprotein molecules, varying both in molecular weight and lipide composition—and probably in their protein moiety as well.[50, 52, 54] They also proposed a nomenclature for the lipoproteins that is followed in this study. Briefly, all lipoprotein molecules that float in a medium of density 1.063 g/ml, commonly called low-density lipoproteins (LDL), are characterized by their rate of flotation in 1.063-g/ml medium in a centrifugal field of 200,000g. This is done by defining a flotation coefficient, called S_f, similar to the sedimentation coefficient of Svedberg, s. In serum, lipoproteins are found with flotation rates varying from $S_f 2$ to S_f 100,000 or greater. Above S_f 1000 these "molecules" are identical with the fat-laden particles known as chylomicra. In addition to the lipoproteins that float at a density of 1.063 g/ml, there are other lipoproteins that sediment at that density. These are termed high-density lipoproteins (HDL). However, they differ from the LDL's in that they do not show a continuous spectrum of densities, but rather have

distinct molecular weights. Furthermore, there are three distinct HDL classes. These are designated HDL-1, HDL-2, and HDL-3. The HDL-1 class has a density of 1.055 g/ml; the HDL-2, 1.075 g/ml; and the HDL-3, 1.145 g/ml. Figure 2 shows the ultracentrifugal patterns of normal human serum lipoproteins. Figure 3 shows the average distribution of the lipoproteins in serum.

From the studies on normal fasting serum, it became clear that the group of lipoproteins with S_f rates in the region S_f 20–400, those in the region S_f 0–20, and the HDL-2, 3 made up natural subgroups and probably had separate functions. Figure 3 shows this subgrouping. These subgroups, which will be called in this paper Class I, Class II, and Class III, also made convenient breaks in the lipoprotein spectrum for the purpose of lipoprotein fractionation. Furthermore, it seemed likely that real differences in chemical composition and structure must exist between these classes. Indeed, studies by Lindgren et al.[55] and Bragdon et al.[56] have borne this out. Figure 4 gives the chemical composition of these classes.

Meanwhile other workers have been trying to elucidate the identity of the protein moiety of the lipoprotein molecule. Shore,[57] Rodbell,[58] and Brown et al.[59] and Avigan et al.[60] have reported on the N-terminal amino acids of human lipoproteins. Shore[57] has also given data on the C-terminal amino acid, as well as total amino acid composition.[57] Figure 5 is a summary of these data. Recent studies on chylomicra have shown that at least part of the protein moiety in chylomicra and HDL's is identical.

Using data such as the lipide and protein composition of the lipoprotein classes, their molecular weight, and their amino acid composition, Lindgren et al.[61] proposed a theory of lipoprotein transformation. In brief, the theory states that HDL's are the primary lipoproteins, or the backbone of the lipoprotein-fat transport system. The chylomicra, S_f 20–400, and S_f 0–20 molecules are aggregates of the HDL lipoproteins and ingested fat. The authors supported the theory on mathematical consideration. The theory accounted for the spectrum of LDL molecules by assuming that a chylomicron is essentially a droplet of triglyceride with a surface layer of HDL molecules to stabilize and solubilize the fat in the serum. Under the action of a lipide clearing factor (heparin-activated), triglycerides are removed from chylomicra, and the S_f 20–400 molecules appear. More triglyceride is removed, and HDL-2, 3 molecules dissociate from the S_f 20–400 molecules, leaving the S_f 0–20 molecules. This class consists of a cholesteryl-ester phospholipide core, a residue from the chylomicra. Small amounts of these compounds are found in chylomicra, surrounded by HDL-1 molecules. This core is finally removed, leaving free HDL-1 molecules. Thus, when fat is eaten, the liver synthesizes HDL molecules which are emulsified with ingested fat, forming the chylomicra, and the LDL are formed by subsequent removal of triglyceride from chylomicra.

The basic concepts of this theory—i.e., the idea of transformation of the lipoprotein through the LDL spectrum—has received support from the work of Scanu and Page,[62] Grundy et al.,[63] and Rodbell and Fredrickson[64] in recent papers.

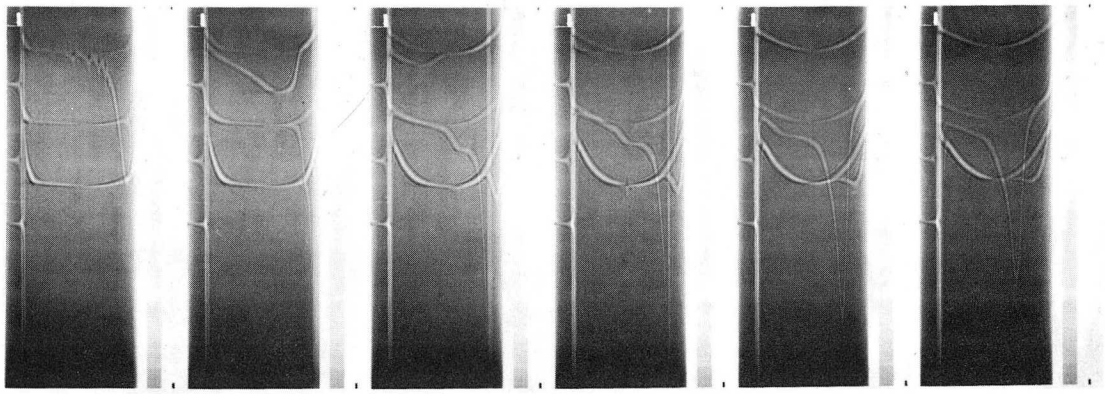

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Fig. 2. Representative schlieren flotation patterns obtained in the ultracentrifuge for the three major lipoprotein classes of human sera. The upper pattern is $S_f20-400$ class floated in a medium of 1.063 g/ml. The middle pattern is S_f0-20 class floated in a medium of 1.063 g/ml. The lower pattern is the HDL-1, 2, 3 class floated in a medium of 1.21 g/ml. Time of each exposure after ultracentrifuge obtained speed of 52,640 rpm, is, from left to right, 0, 6, 22, 30, 48 and 64 minutes respectively. This run was made with three double-sector-type ultracentrifuge cells in a Spinco series ANC-1 four-cell rotor.

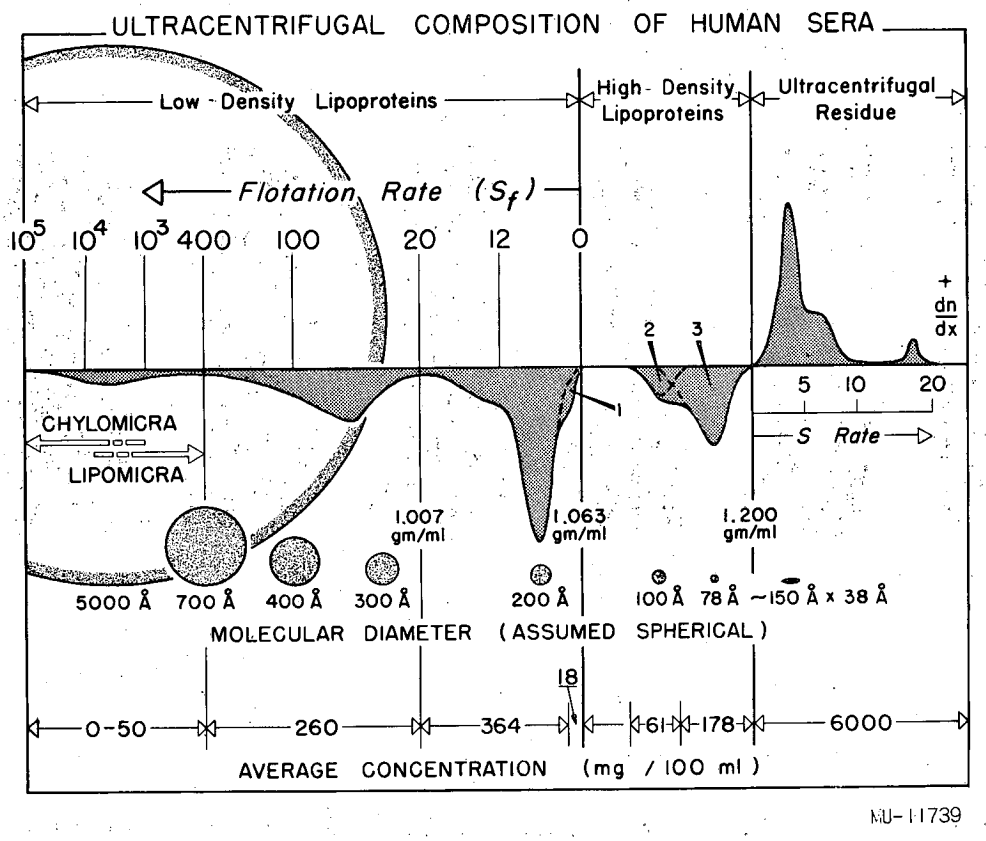

Fig. 3. The ultracentrifugal composition of human serum, showing relative molecular size as well as average serum concentration in 45-year-old males. The nonlipoprotein residue is plotted with a 30-fold scale reduction on the dn/dx scale.

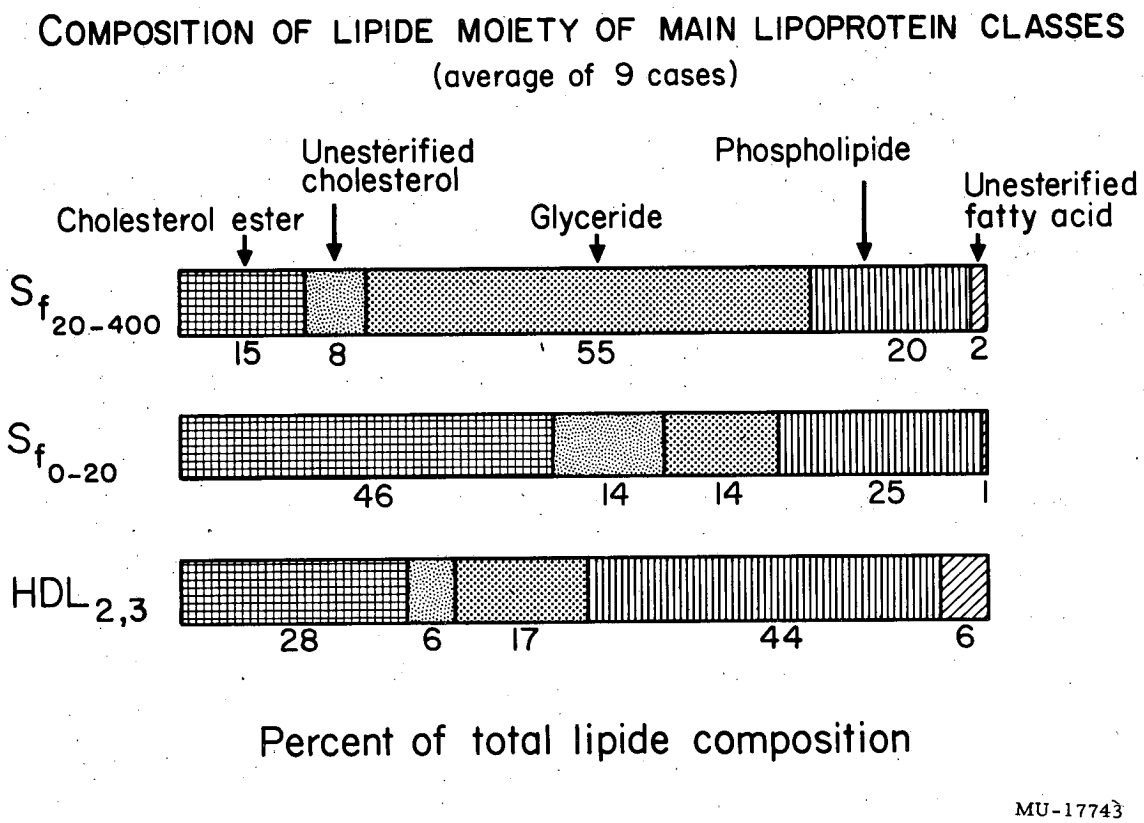

Fig. 4. The average lipide composition of nine individuals is given with respect to the three major lipoprotein classes. The phospholipides are presented as a single fraction.

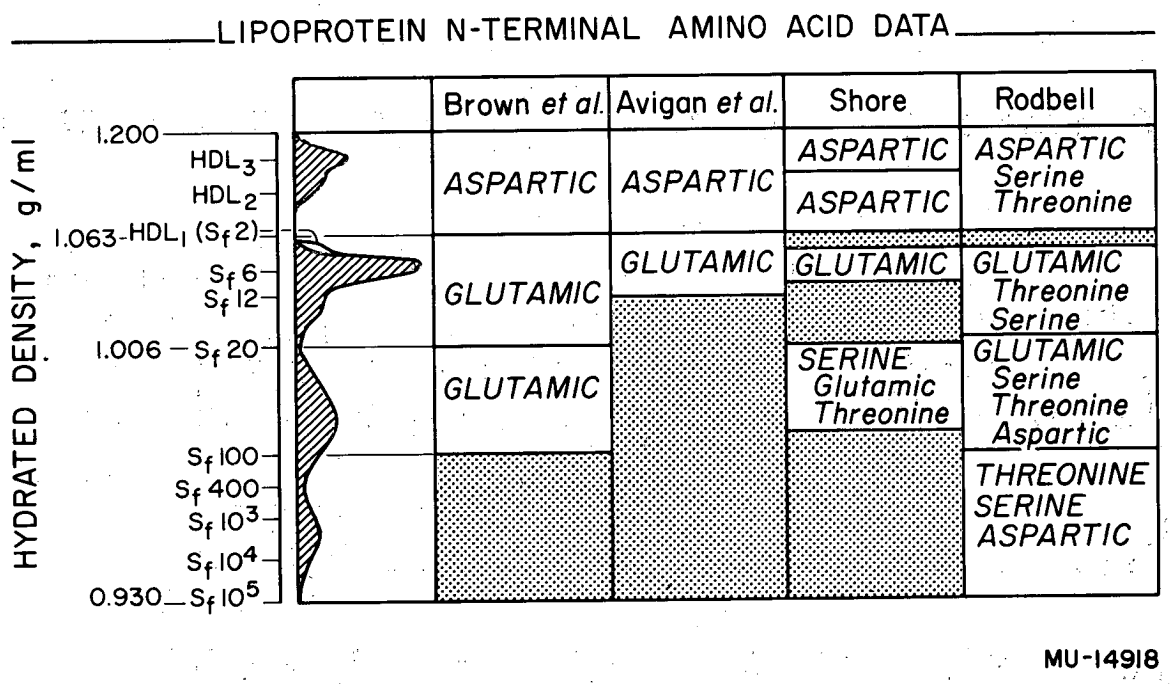

Fig. 5. Major N-terminal amino acid composition of the serum lipoproteins. The rectangles are placed to indicate which lipoprotein bands within the total lipoprotein distribution were studied. Dominant components are shown in the larger print. Trace components are not shown.

In the theory as originally proposed by the authors, certain anomalies remain unanswered. There is too much phospholipide, cholesterol ester, and protein in the chylomicra to be readily accounted for in the breakdown products. At low S_f values, the model was inadequate because removal of triglyceride would not provide a composition that agreed with observation. It was also difficult to reconcile the lipide-to-protein ratio in the LDL's and HDL's. These and other problems of lipoprotein composition are discussed further, in the section that presents a modified theory of lipoprotein transformations and fat transport, in light of the data presented in this paper as well as in other recent works.

EXPERIMENTAL PROCEDURES

The experimental procedure followed in this study consisted of four separate procedures, one of which was developed essentially for this study, namely the isolation and identification of the phospholipides by chromatography and infrared spectrophotometry.[30] The remaining procedures were adaptations or modifications of previous works. They involved ultracentrifugal separation of serum lipoproteins, organic-phase extraction of lipides from serum, and gas chromatography of methyl esters of fatty acids from phospholipides. These procedures are described in the order in which they were applied to the samples studied. All analyses were performed in an identical manner.

Sampling Procedure

Blood was drawn in 250-ml quantities from donors on whom previous routine serum lipoprotein analyses had been performed as part of routine physical examinations. All donors were employed at the Livermore site of the Lawrence Radiation Laboratory. Subjects with high serum lipide values were selected for study so that adequate quantities of the respective serum phospholipides would be obtained. Although it was realized that this would prejudice the data, it was considered necessary because of limitation on the amount of blood obtainable.

The blood was drawn from the subjects a minimum of 3 hours after breakfast. This is not considered a fasting sample, but rather postabsorptive. However, no visible lipemia was present in any case, and the S_f 20–400 lipoproteins were not elevated above these subjects' normal levels. See Appendix I for ultracentrifuge flotation patterns. The presence of a few molecules with S_f rates faster than 400 is considered normal[54] and expected, especially when an individual has an A. I. above 100. (A. I. is a function developed in this laboratory (see Ref. 53, 54) by applying statistics to the observed serum lipoprotein levels in humans for the assessment of the relative importance of the S_f 0–12 and S_f 12–400 lipoprotein levels in the incidence of myocardial infarcts.
It is defined by the following equation:

$$A.I. = 0.1\ (S_f\ 0\text{–}12\ \text{conc.}) + 0.16\ (S_f\ 12\text{–}400\ \text{conc.})$$

The choice of the decimal point in the factors 0.1 and 0.16 is arbitrary. This function is a convenient measure of the total LDL concentration.)

Blood was drawn at approximately 10 a.m. and allowed to clot by standing for 6 hours at room temperature. At this time the clot was removed and the remaining cells removed by centrifugation at 2000 rpm for 20 minutes in an International model L centrifuge with a number 250 rotor. The serum, usually slighly hemolyzed, was then transferred to 6-ml Lustron preparative ultracentrifuge tubes. The filled tubes were placed in a Spinco series 12-G rotor, designed for rotation at 40,000 rpm.

Ultracentrifugation

The method of separation of the serum lipoproteins was developed in this laboratory by Drs. Frank Lindgren and Alex Nichols, and has not been previously described in a publication. It is a system utilizing NaBr solutions containing a constant content of NaCl, (0.195 molal), but presents no radical departure from the standard lipoprotein separations[65] by flotation in the preparative ultracentrifuge. All centrifugations were carried out in a Spinco Model L ultracentrifuge at 20°C.

The unaltered serum was spun for 24 hours at 40,000 rpm. Unaltered serum has a density of 1.006 g/ml. At this density the result of centrifugation of the unaltered serum for 24 hours at 40,000 rpm is that the top milliliter in the preparative tube contains lipoproteins floating with S_f rates greater than 20. All material denser than 1.006 g/ml sediments to the lower 3 ml of the tube. The second ml is void of all macromolecules. The top ml was collected in a vial and placed under refrigeration at 4° C to prevent denaturation of the lipoproteins until they could be extracted. The second ml was collected in the same manner. A special pipetting technique developed in this laboratory was used for the removal of fractions from the preparative tubes.[66] The remaining 4 ml of serum in the preparative tube was brought to a density of 1.070 g/ml by the addition of 2 ml of a previously prepared solution of NaBr. The tubes were then respun for 24 hours at 40,000 rpm. The top ml of the preparation at a density of 1.062 g/ml contains all lipoproteins with flotation rates greater than $S_f 2$. The denser materials sediment to the bottom 3 ml of the preparative tube while the second ml from the top is again free of macromolecules. The top ml and the second ml were pipetted into separate vials and stored at 4°C, as in the previous run. To the remaining 4 ml was added 2 ml of a previously prepared NaBr solution which brought the density of the solution to 1.218 g/ml. The tubes were then respun for 36 hours at 40,000 rpm.

Following the final centrifugation the top ml of the preparatory tube at a density of 1.203 g/ml contains the high-density lipoproteins (HDL-1,2,3), the second ml is clear of macromolecules, and the lower volume contains the serum residue consisting principally of serum albumin and globulins. After this recentrifugation, the top and second ml were collected and stored as described before. The serum residue was discarded. The high-density lipoproteins obtained in this manner are the HDL-1, 2, 3. However, HDL-1 is considered (see below) to be closely related chemically to the S_f 0–20 (Class II) lipoproteins.[61] Thus, it was considered desirable to remove the HDL-1 from the HDL-2, 3 molecules. To do this, the top ml of the HDL isolation step was diluted to a density of 1.075 g/ml and respun at 40,000 rpm for 48 hours. This procedure floated the HDL-1 to the top ml of the preparative tube, while the HDL-2, 3 fraction was concentrated in the bottom ml of the tuble. The intermediate 4 ml was generally clear of macromolecules. For analytical purposes, however, the top ml was collected in one vial, the next two in another, the fourth and fifth in another, and the bottom ml in still another so that analytical runs could be made to determine the exact distribution of the lipoproteins.

For exact characterization of the lipoprotein fractions obtained in this procedure, all collected cuts from the preparative runs were analyzed

by ultracentrifugation in a Model E Spinco analytical ultracentrifuge (see Appendix I). The low-density lipoproteins were run in a solution of 1.063 g/ml density. The HDL's were run in a solution of 1.21 g/ml density. The resulting schlieren photographs are of flotation patterns. In the unaltered-serum run, the density was brought to 1.063 g/ml by addition of solid NaBr to the solution. The S_f 0–20 class was run as obtained from the preparative run, as was the HDL-1, 2, 3 fraction. The separated HDL-1 and HDL-2, 3 samples were brought to a density of 1.120 g/ml by addition of solid NaBr.

The analytical runs were not calculated exactly to obtain values for lipoprotein concentrations in the serum. However, a rough estimate was obtained in order to judge the size of aliquot to be used for extraction of lipides. The solution was diluted to an appropriate value so that a 10-ml aliquot would contain 100 mg of lipides. The extraction procedure and column chromatography to be described have been previously published in greater detail;[30] what follows is only a summary of the procedure. For further details see the original publication.

Extraction

All solvents were reagent grade. The purity was checked by recording the infrared spectra of their nonvolatile residues. The extraction method used was a modification of the procedure of Sperry and Brand,[67] which was based mainly on the work of Folch et al.[68] A 10-ml aliquot of a lipoprotein fraction was pipetted into a 250-ml volumetric flask containing 83 ml of methanol. Then 83 ml of chloroform was added to the flask, and the flask was placed in a 60°C water bath for 15 minutes. The flask was then cooled in tap water until it returned to room temperature and brought to volume with chloroform. The precipitated protein was removed by filtration through a fast filter paper into a 250-ml graduated cylinder. The volume recovered after the filtration was recorded and future calculations corrected for the amount lost in filtration. The filtered extract was brought to 250 ml with chloroform and transferred to a 500-ml separatory funnel (which was fitted with a Teflon stopcock), and 50 ml of distilled water was added to the funnel. It was shaken vigorously for 5 minutes, then allowed to stand until complete separation of both phases was achieved (this usually took 1 or 2 hours).

The lower (organic) phase containing the lipides was drawn off; the aqueous phase was discarded. The organic phase was transferred to a 250-ml distillation flask, and the solvent removed with a rotary evaporator under reduced pressure. When most of the solvent was removed, the residue was transferred, with chloroform, to a tared vial and the remaining solvent removed by blowing nitrogen over the surface. The vial was placed in a vacuum desiccator for 24 hours and then weighed.

Chromatography

The adsorbent was prepared by sifting and mixing together thoroughly two parts (by weight) of precipitated silicic acid (Fisher Scientific Co.) and one part celite (analytical filter aid, Johns-Manville and Co.). The mixture was washed with methanol, dried, and finally heated to 120°C for 24 hours. It was then stored in a vacuum desiccator.

The column was prepared on the basis of 10 to 15 mg of phospholipide to 1 g of silicic acid-celite mixture. The column material was added as a slurry in methylene chloride and packed by air pressure from a syringe fitted to the top of the column. Gentle tamping with a glass rod was used to prevent channeling and to provide a level surface. The column was washed with approximately ten volumes of methylene chloride before addition of the lipides to the column. (A column volume is approximately 2.5 ml per g of silicic acid-celite).

The lipide extract was added to the column in 2 ml of methylene chloride. Care was taken not to disturb the level surface of the column and to see that there was no channeling present. The lipides were washed onto the column with another 3 ml of methylene chloride, and then the liquid level was brought to the surface of the silicic acid. Again, care was taken not to allow the liquid level to fall below the silicic acid level at any time during the chromatography. After addition of the sample to the column, the elution was carried out according to the scheme shown in Table I.

Table I

Silicic acid chromatographic scheme for a 2-g column, silicic acid-celite mixture, 2:1 by weight

Fraction	Solvent	Volume (ml)	Components
I	CH_2Cl_2	100	Nonphospholipides (cholesterol, cholesteryl esters, glycerides, fatty acids)
II	Acetone	20	Principally nonphospholipides (oxidized?), pigments, and trace of phospholipides
III	35% methanol 65% CH_2Cl_2	10	Phosphatidyl ethanolamine plus phosphatidyl serine and small amounts of nonphospholipides
IV	35% methanol 65% CH_2Cl_2	25	Lecithins
V	95% methanol 5% H_2O	50	Lecithins and sphingomyelins

Fractions II, III, and IV were small enough to collect in 25-ml screw-cap vials which had been previously tared. These fractions were evaporated to dryness under nitrogen on a hot plate at 50°C, and then desiccated for not less than 6 hours before weighing. Fractions I and V were collected in 150-ml beakers, evaporated with nitrogen until the volume was sufficiently reduced, and then transferred to vials of the same type as described for Fractions II, III, and IV. After desiccation, weights were obtained on each fraction, mainly for correlation with the infrared data.

Fraction I contained virtually all of the nonphospholipides, or 70 to 80% of the total serum lipides. Fraction II was very small in amount, serving mainly to remove residual nonphospholipides, with acetone acting as a scavenger for oxidized materials, etc. Traces of phospholipides amounting to less than 1% of the total were usually found in Fraction II. The first two fractions were weighed only for inclusion in total recovery figures.

Fraction III consisted mainly of phosphatidyl ethanolamine plus phosphatidyl serine, with small amounts of nonphospholipides still persisting.

Fraction IV contained 60 to 80% of the lecithin present in the total extract. It was usually quite colorless and free from contamination.

The remaining lecithin was eluted together with sphingomyelin in Fraction V. It was not possible to separate sphingomyelin free of lecithin by this procedure. However, it was not necessary to do so for the purpose of infrared spectroanalysis. Indeed, Fractions IV and V could have been analyzed as a single mixture, but it was desirable to remove enough of the lecithin so that the concentrations of the two components were more nearly comparable.

Infrared Measurements

After collection of the chromatographically separated phospholipide fractions, the quantitative analysis was performed by infrared spectrophotometry. Also chemical phosphorus determinations were performed as a check on the method; the ancillary data are shown in Appendix II.

All measurements were made with a Baird Associates double-beam recording infrared spectrophotometer equipped with a sodium chloride prism. The absorption cell was designed in this laboratory and has an optical path length of 0.9 mm and a volume of 0.15 ml.

Fractions I and II were not included in the infrared determinations.

Fraction III usually contained between 1.0 and 2.5 mg of material and was dissolved in 0.20 to 0.375 ml of carbon disulfide for recording its spectrum. This was done by transferring the sample in a minimum volume to a small, graduated centrifuge tube. The volume was then brought up to the desired level and 0.15 ml was transferred to the absorption cell. A preliminary gravimetric weight on each fraction was useful

here, in that the optimum concentration for a 0.9-mm cell is between 5 and 7.5 mg/ml. Weights of from 1.0 to 1.5 mg were diluted to 0.20 ml and weights from 1.5 to 2.5 mg were diluted to 0.375 ml.

Fraction IV usually contained between 15 and 20 mg, and was transferred to a 3-ml volumetric flask with carbon disulfide and was made up to volume. If the weight was lower a 2-ml flask was used.

Fraction V was dissolved in an appropriate amount of chloroform, usually 1.0 ml, because carbon disulfide does not transmit in the necessary spectral region and sphingomyelin is relatively insoluble in carbon disulfide.

The spectrum of each eluted fraction was recorded from 5 to 11 microns (μ). Over the bands whose absorbances were to be measured the scanning rate was kept slow enough to allow full response in the recorder. A cell containing pure solvent was used in the reference beam, and for the desired precision, it was necessary to run background curves of solvents contained in the sample cell.

Typical spectra obtained from the chromatographic studies of a lipoprotein extract sample are shown in Fig. 6. The region from 6.0 to 7.2 μ is obscured in carbon disulfide solutions, and the regions from 6.4 to 6.7, 7.8 to 8.7, and 9.3 to 9.9 μ are obscured in chloroform. The dotted line is the curve for solvent vs solvent background.

Gas Chromatography

For the purpose of gas chromatographic analysis, the fractions recovered after the infrared runs were subjected to a transmethylation procedure in order to obtain the methyl esters of the constituent fatty acids.

Approximately 5 to 10 mg of phospholipides was placed in a 10-ml glass-stoppered tube. Then 8 ml of a 1% by weight solution of H_2SO_4 in methanol was added. The tube was then tightly stoppered and heated at 75°C for 2 to 3 hours. At this time it was removed from the heat and the volume of methanol reduced by evaporation with nitrogen to 1 ml. The methanol was transferred to a small separatory funnel (with a Teflon stopcock) and 4 ml of distilled H_2O added. The solution was neutralized with an excess of solid $NaHCO_3$ and extracted four times with 10 ml of petroleum ether (30°–60° boiling range). The petroleum ether phases were pooled and evaporated to dryness with nitrogen. The collected methyl esters were placed in a 2-ml vial and dissolved in a known amount of n-hexane (purified by redistillation). The vial was then securely stoppered.

The esters were applied to column in .005-ml aliquots of the hexane solution by means of a Beckman micro injector. The gas chromatographic unit was designed and built in this laboratory, and uses an ionization detector of the type designed by Dr. Lovelock[38] (using a Sr^{90} source of β particles). The stationary phase was LAC-728 (a succinic acid polyester of diethylene glycol obtained from Cambridge Industries Co., Inc., Cambridge, Mass.) absorbed on Chromosorb (obtained from Wilkens Instruments and Research, Inc., Walnut Creek, California) 48–65 mesh.

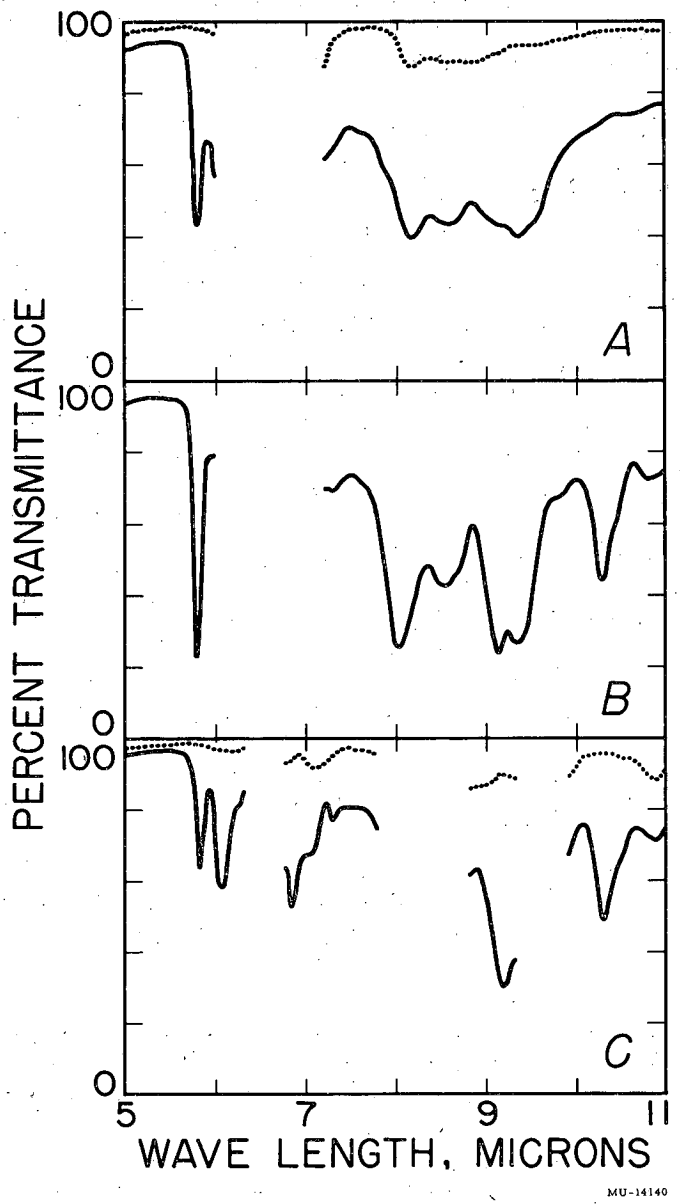

Fig. 6. Typical infrared spectra obtained from chromatographic eluates of a serum lipoprotein fraction. The region from 6.0 to 7.2 μ is obscured in CS_2 solution and from 6.4 to 6.7, 7.8 to 8.7, and 9.3 to 9.9 μ in chloroform. The dotted line is the solvent base line.
A, Fraction III, phosphatidylethanolamine and phosphatidyl serine, 3.79 mg in 0.5 ml of carbon disulfide;
B, Fraction IV, lecithin, 8.00 mg/ml in carbon disulfide;
C, Fraction V, lecithin and sphingomyelin mixture, 5.63 mg/ml in chloroform.

The 4-foot column was maintained at 190°C, and the carrier gas was argon at a flow rate of 80 to 90 ml per minute. Typical gas chromatograms obtained from this column are shown in Fig. 7.

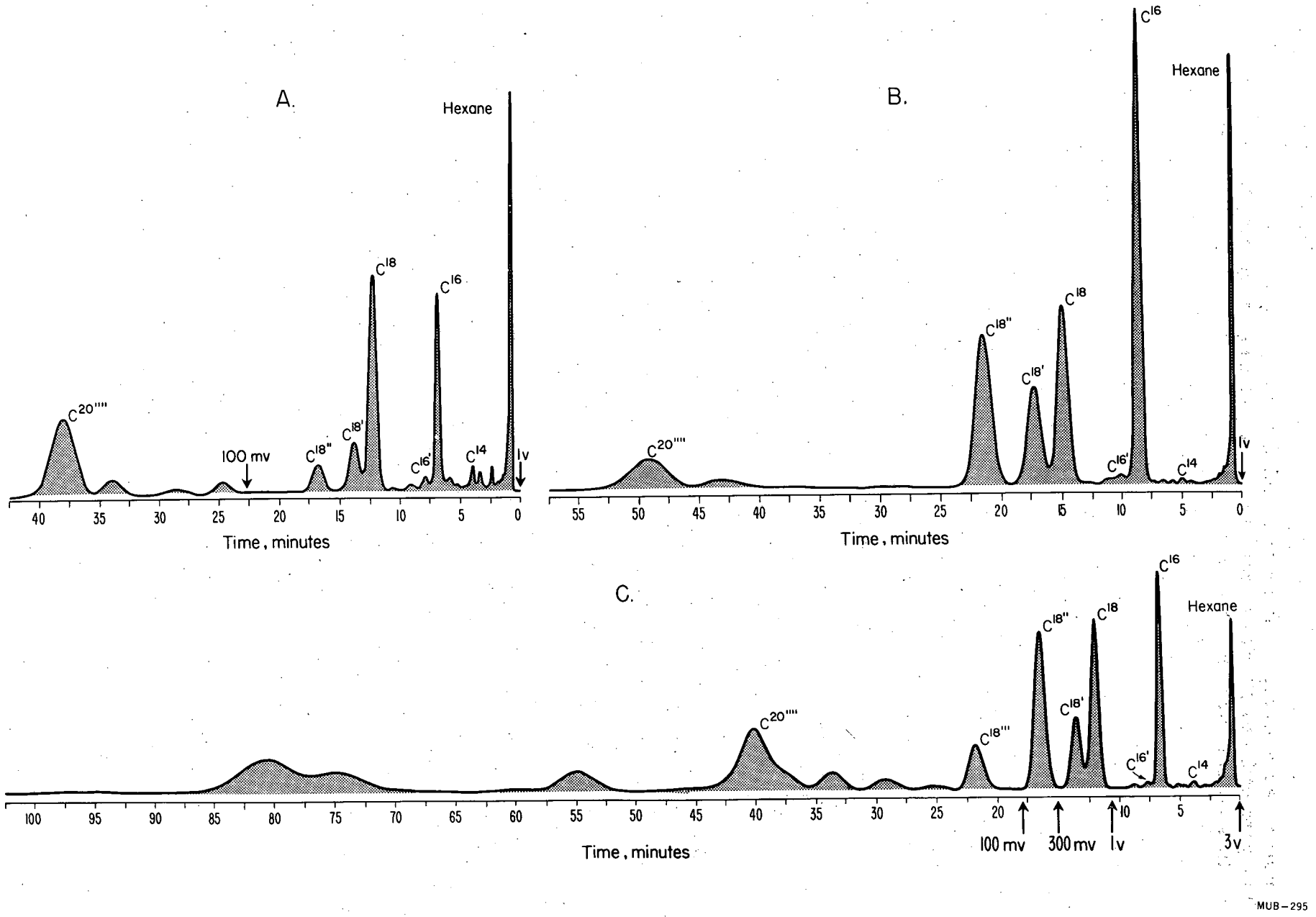

Fig. 7. Gas chromatograms of fatty acid methyl esters from serum phospholipides. Operating conditions identical to those described in Fig. 9. Curve A: S_f0–20 lecithin fatty acid esters. Curve B: Total serum phosphatidyl ethanolamine– serine fatty acid esters. Curve C: Total serum sphingomyelin fatty acid esters. Recorder sensitivity changes are as indicated.

METHODS OF CALCULATION

The quantitative determinations of the individual phospholipides were made from the infrared data in the manner described below. Only the chromatographic fractions III, IV, and V were analyzed in this procedure. The calibration procedure, which is critical in the calculation, will be described first. Any error induced by calibration will be systematic and tend to invalidate the final results.

Calibration

The infrared spectra of the reference compounds are shown in Fig. 8. Figures 8 A and B are phosphatidyl ethanolamine and lecithin, respectively, in carbon disulfide, and Fig. 8 C is sphingomyelin in chloroform. Figure 8 D is sphingomyelin on NaCl plates, showing the full spectrum unobscured by solvent absorption.

The materials used as standards were natural products, purified as far as practical by chromatography. As further criteria of purity, the infrared spectra and chemically determined phosphorus values were used. Material for calibration of Fraction III was prepared from acetone-insoluble lipides of egg yolks by rechromatography, according to the procedure of Lea, Rhodes, and Stoll.[24] Lecithin, obtained from serum in the chromatographic scheme presented here (Fraction IV), was further rechromatographed three times for use as a standard. These substances so prepared were considered acceptable if their phosphorus content[69] was 4.0 ± 0.1%, and their infrared absorptions at 10.3 μ were minimal and maximal, respectively. Fifth fractions from scaled-up serum phospholipide chromatography, or pooled fifth fractions from routine serum phospholipide chromatography, served as a source of sphingomyelin. For purification, they were subjected to mild basic hydrolysis as described by Hack;[2] then the extract from the neutralized hydrolysis mixture was rechromatographed by the usual procedure. Fraction V was taken as a standard for sphingomyelin if its phosphorus content was satisfactory and no ester carbonyl absorption at 5.8 μ in the infrared spectrum could be detected.

Although synthetic compounds of rigorous purity would be desirable as standards, most of those that have been prepared so far (by Professor E. Baer, Toronto) have been compounds containing saturated fatty acids. These have very limited solubility in carbon disulfide when the fatty acid chain lengths correspond to those expected to occur in natural phospholipides. When some synthetic dioleoyl lecithin was made available (courtesy of Professor Baer), absorptivities at the major peaks agreed within 2 to 3% except at 5.8 μ, where the coefficient for serum lecithin was about 10% lower than the synthetic. This presumably reflects a lower content of ester groups per gram, and could arise from a difference in fatty acid size or possibly from the presence of lysolecithin or some unknown contaminant in the natural material. In the absence of more definite information about the fatty acid composition of serum phospholipides and the fluctuations thereof, empirical data were used for the natural substances, subject to the criteria stated above.

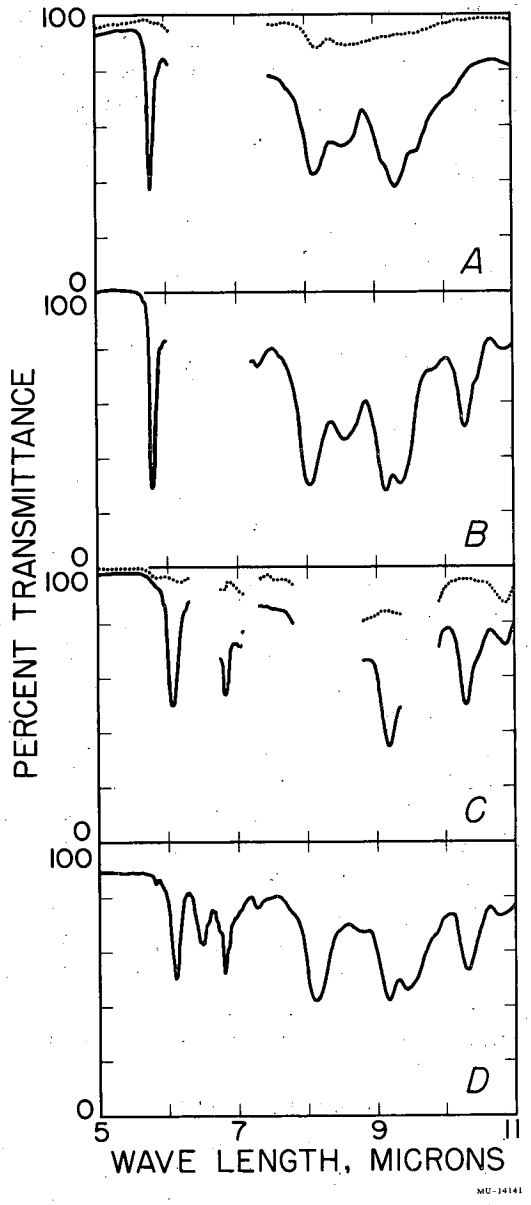

Fig. 8. The infrared spectra of reference compounds.
A, phosphatidyl ethanolamine, 5.06 mg/ml in carbon disulfide;
B, lecithin, 6.55 mg/ml in carbon disulfide;
C, sphingomyelin, 6.06 mg/ml in chloroform;
D, film of sphingomyelin on NaCl plates.
All reference compounds in this figure were prepared as indicated in the text.

Table II lists a set of typical absorption coefficients of the measured phospholipides at their principal absorption bands. The absorption coefficients, designated α, are absorbances per unit concentration (mg/ml) in an absorption cell of 0.9 mm optical path length. The phosphatidyl ethanolamine and lecithin samples were dissolved in carbon disulfide, and the sphingomyelin was dissolved in chloroform. These values, of course, apply only to the infrared spectrometer in this laboratory.

Table II

Absorption coefficients, α, for the principal absorption bands of the major serum phospholipides

Wave length (μ)	Absorption coefficients, α, in absorbancy per mg/ml		
	Phosphatidyl ethanolamine in CS_2	Lecithin in CS_2	Sphingomyelin in $CHCl_3$
3.4	0.126	0.122	0.139
5.8	0.077	0.080	0.003
6.1		0.001[a]	0.047
6.8			0.038
8.1	0.060	0.073	
9.2		0.074	0.063
9.3	0.073	0.070	
10.3	0.020	0.044	0.045

[a]This value is obtained in chloroform; this region is obscured in carbon disulfide. These absorption coefficients apply to concentrations within the range 5.0 to 7.5 mg/ml, over which the calibration curves are quite linear. For concentrations above this range the validity of these α's is questionable.

Calculation of Phospholipides

Fraction III (Fig. 6 A) is the phosphatidyl ethanolamine-serine fraction. The concentration was determined by calculating the absorbancy at 5.8 μ, using the experimentally determined absorption coefficient as determined above to convert absorbancy into mg/ml. The rest of the curve was used primarily to indicate the purity of the sample. The absence of a 10.3-μ band showed that no lecithin was present. The 9.3-μ band could also have been calibrated, but this band had considerably more interference from contaminants than the 5.8-μ band.

Fraction IV (Fig. 6 B) was calculated as lecithin, and usually the 9.2- or 10.3-μ band was used to calculate the concentration of the lecithin present. The 5.8-μ band was found to be less reliable.

Fraction V (Fig. 6 C) was a two-component mixture containing both lecithin and sphingomyelin. The absorption curves of lecithin and sphingomyelin are very similar beyond 7 μ (see Fig. 8), but they differ significantly in the 5.8-to-6.2-μ regions, as seen in the chloroform-solution spectrum. By using these absorption bands at 5.8 and 6.1 μ and two standard simultaneous equations, it was possible to calculate the amounts of lecithin and sphingomyelin present. The absorption of each compound at 10.3 μ is almost identical, and this band provided a convenient check on the total weight of material in the fraction. The weight calculated at 10.3 μ should agree with the sum of the weights given by the two simultaneous equations.

Briefly, the mathematics of the simultaneous equations are given in this manner.[70] An equation for the absorption at 5.8 μ and a similar equation for the absorption at 6.1 μ were set up, thus:

$$A_{5.8} = a_L^{5.8} C_L + a_S^{5.8} C_S , \qquad (1)$$

$$A_{6.1} = a_L^{6.1} C_L + a_S^{6.1} C_S . \qquad (2)$$

As the values of a were known for the absorption cell used, these two equations were rearranged to yield concentration in terms of the absorbancies and four new constants:

$$C_1 = k_1 A_{5.8} + k_2 A_{6.1} , \qquad (3)$$

$$C_S = k_3 A_{5.8} + k_4 A_{6.1} . \qquad (4)$$

When typical values were substituted for the constants, k, the equations became

$$C_L = 11.25 A_{5.8} - 1.5 A_{6.1} , \qquad (5)$$

$$C_S = -2.25\ A_{5.8} + 22.5\ A_{6.1}\ . \qquad (6)$$

Since the volumes of the solutions were known, concentrations were converted to weights by simple multiplication. The weight of lecithin determined in Fraction V by Eq. (5) was then added to that determined in Fraction IV to give the total amount of lecithin present. At times a similar correction was applied to Fraction IV, if sphingomyelin was suspected in this fraction. The total amount of sphingomyelin was usually, however, simply that calculated by Eq. (6).

The amounts of individual phospholipides were calculated as percentage of the total phospholipide.

Calculation of Fatty Acids

The fatty acid compositions of the individual phospholipides were derived by triangulating the resultant areas of the elution peaks as recorded on the readout recorder of the gas chromatographic unit, a Brown recorder. The linearity of the response was previously proven and the unit calibrated by passing known amounts of highly purified fatty acid methyl esters (obtained from the Hormel Foundation) through the column. See Fig. 9 for the curve of a standard mixture run on the column. It was found that the total area obtained by summing the areas of individual peaks was adequate to represent the total amount of material added to the column if corrections were applied for differences in molecular weight of the different fatty acid methyl esters. This is in agreement with the observation by Lovelock that the response of a β-ionization detector is linear with number of organic molecules present in the carrier gas.[38] Thus the percentage of any of the various fatty acid methyl esters present was obtained by dividing the corrected area of the individual elution peak into the corrected total area of the chromatogram.

Time sequence of the elution peaks from the column was taken as the criterion of identification of the particular fatty acid methyl ester.[39] The elution characteristics of the column were previously determined by the same standardization procedure which was used for calibration of the column with standard methyl esters. A semilog plot of elution time versus carbon length for saturated fatty acid methyl esters is seen in Fig. 10, as well as a plot for degree of unsaturation in the normal-chain C-18 series: oleic, linoleic, linolenic. Notice the striking linearity of the curves.

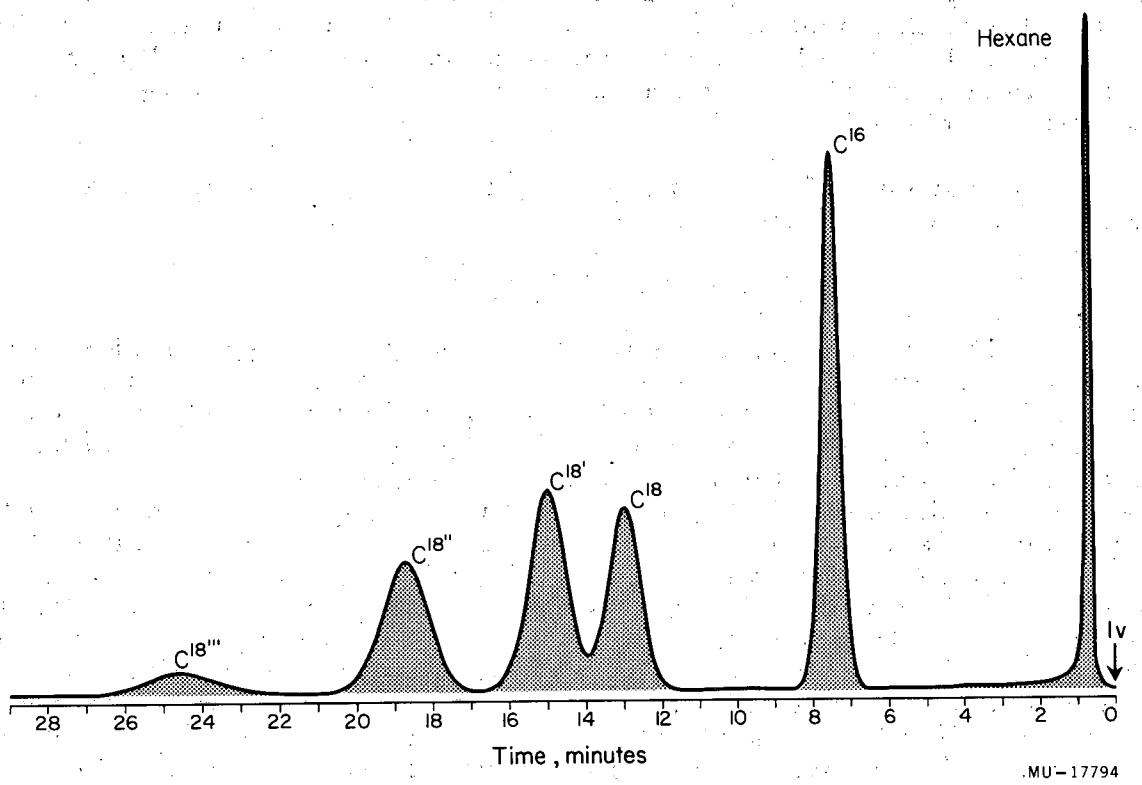

Fig. 9. The elution curve of a known mixture of methyl esters of fatty acids obtained from the Hormel Foundation. The stationary phase was Lac-728 maintained at 190°C on a 4-foot column. The flow rate was 80 ml of argon per min. Samples were injected in n-hexane solution. Total sample weight, 80 μg. Recorder sensitivity was 1 volt, full scale.

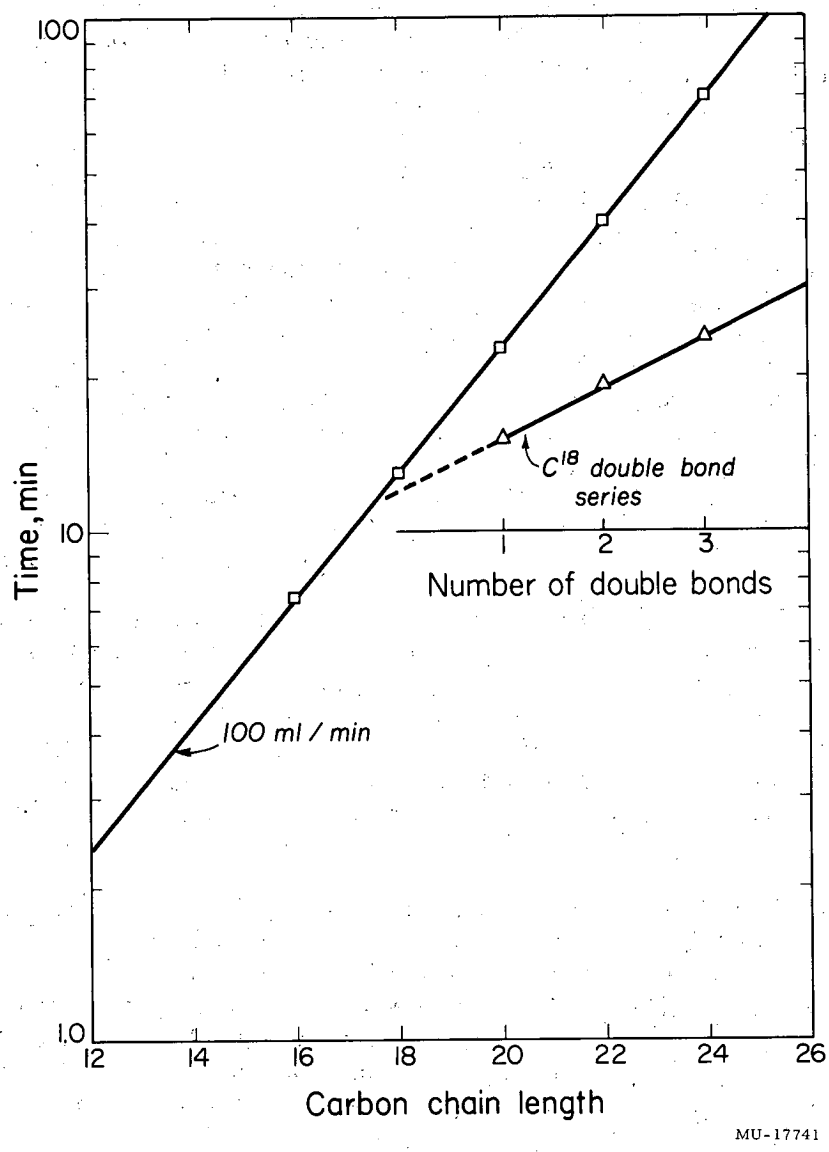

Fig. 10. A semilog plot of retention time of the methyl ester of the saturated fatty acid extending from C^{12} to C^{26} versus number of carbon atoms in the chain. Operating conditions identical to those described in Fig. 7 except that flow rate was 100 ml/min. Also shown is log of retention time of the C^{18} unsaturated methyl ester series as function of double bonds present in the molecule.

PRESENTATION OF RESULTS

The results are presented in tabular form and converted to percentages whenever possible. Important values obtained from the literature have been included wherever necessary for comparison. The chromatographic data are given in Appendix II.

Table III shows the average individual values of the total serum phospholipides obtained in 10 normal males (A series) in an earlier study related to the development of the analytical method. These data were obtained on whole serum that had not been fractionated ultracentrifugally into lipoprotein classes. Also included in this table are the total serum phospholipide values for the five cases (B series) on which the three lipoprotein classes were analyzed separately. These values are reconstituted from the values obtained on the separate lipoprotein classes by using average values for concentration of the lipoproteins in their sera. Although there is considerable individual variation, the average values for the A series and the B series agree quite well. It should be noted that average A. 1. of the A series is 50 points lower than that of the B series, but no discrepancies appear in the phospholipide distribution. This is discussed further below.

Table IV presents the average values of the A and B series, along with the values from Hack[20] and Phillips[21] taken from the literature. In Hack's data, mg has been converted to percentage. Phillips reported values for lysolecithin as well as for the components reported in this work. For purposes of comparison, his percentage of lysolecithin has been added to his lecithin fraction. In general the agreement is good, and the variance is less than that found between individuals. There appear to be no differences on the basis of sex.

Table V presents the results of this study on the phospholipide distributions of the three lipoprotein classes. The percentage of phospholipide of the total lipide extract in each of the three classes is remarkably constant. Only in the HDL-2, 3 is there any difference that may be greater than experimental error. This may be because the HDL-2 and HDL-3 molecules have different percentages of phospholipides and the concentration of each is independently variable. The distribution of the phospholipides is not identical in major serum lipoprotein classes. In Class I, which contains less than 20% of the total serum phospholipides, the ratios of the individual phospholipides closely approximate the distribution of normal total serum, with the phosphatidyl ethanolamine-serine fraction being slightly high. Neither Class II or III appears similar to the total distribution. In Class II, which contains 51% of the total serum phospholipide, is found more than 65% of sphingomyelin in serum, and only 45% of the total phosphatidyl ethanolamine-serine fraction. Even more striking is the low value of sphingomyelin in Class III, which has 31% of total phospholipides but only 20% of the sphingomyelin. Figure 11 gives the distribution of the phospholipides in the three fractions as per cent of the total amount of that phospholipide in serum.

Table III

Distribution of total serum phospholipides

Run No.	Subject	Age	Sex	Total lipide (mg/ml)	A.I.	% PL of TLE	Phospholipides (% of the total phospholipides) PE-S	L	S
1A	RH	-	M	8.60	106	24.9	4.9	76.8	17.4
2A	JD	46	M	6.50	54	26.4	6.4	73.4	20.1
3A	JB	34	M	4.40	45	28.4	5.9	71.0	23.1
4A	MM	33	M	3.69	38	29.8	6.5	75.6	17.9
5A	GC	50	M	6.08	64	29.9	5.4	70.4	24.1
6A	LS	37	M	6.48	59	29.6	5.0	76.6	18.4
7A	MV	55	M	6.79	86	28.2	4.6	71.5	23.9
8A	JK	34	M	8.24	57	30.1	7.0	82.6	10.4
9A	MF	40	M	6.49	82	29.2	6.7	73.1	20.2
10A	WC	46	M	4.83	56	33.0	8.4	73.3	18.3
Average Values, A Series					64	29.0	6.1	74.4	19.4
1B	LM	37	M	10.80	144	28.8	5.5	73.1	21.4
2B	FD	37	M	13.07	131	30.1	7.6	71.4	21.0
3B	HG	39	F	9.14	107	33.4	7.6	72.6	19.9
4B	DG	49	F	8.33	88	26.3	5.7	74.6	19.7
5B	RG	48	M	8.94	107	26.9	6.1	75.0	19.0
Average Values, B Series					116	29.1	6.5	73.3	20.8

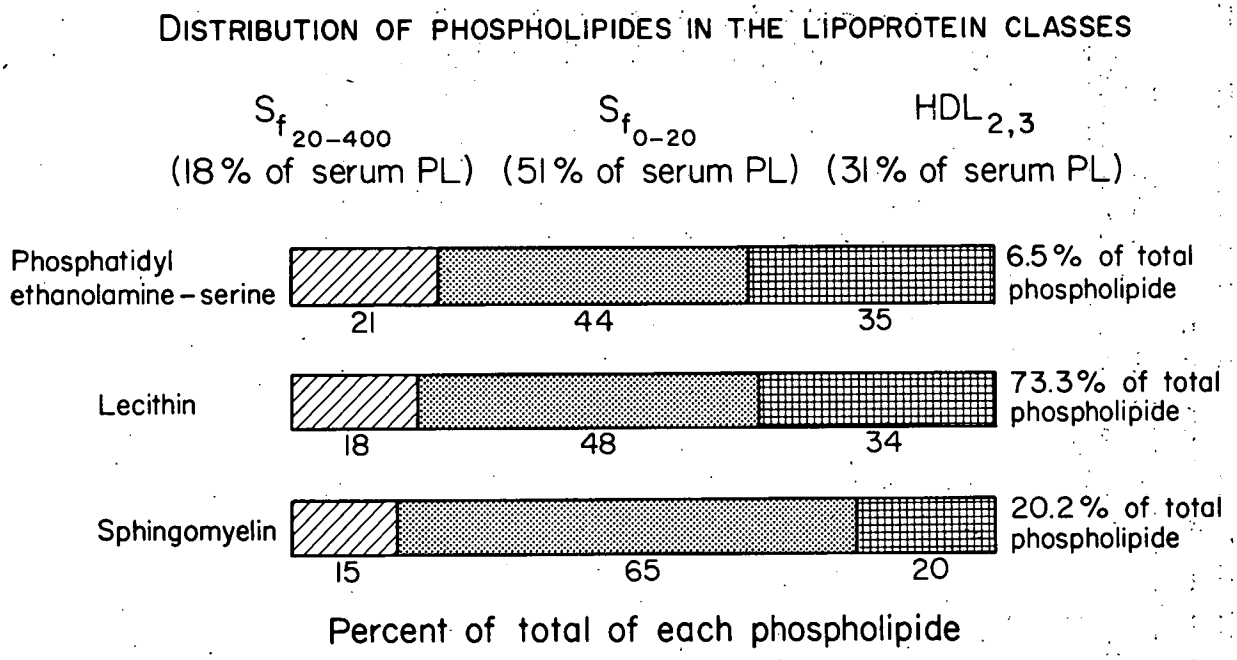

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Fig. 11. The distribution of the individual phospholipides of human sera in the lipoprotein classes in per cent of the total amount of that phospholipide in sera.

Table IV

Comparison of average results obtained in this study with literature values

Source of information	% of total phospholipides			Number of cases studied
	PE-S	L	S	
Hack	5.2	78.8	16.0	8
Phillips	4.6	76.2	19.0	7
This work:				
A Series	6.1	74.4	19.4	10
B Series	6.5	73.3	20.2	5
Average of all cases	5.6	75.7	18.7	30

Table V

Summary of data for serum lipoprotein phospholipides

	Run No.	% of total phospholipides PE-S	Lecithin	Sphingomyelin	% of PL of TLE
Class I, S_f 20–400	1B	7.3	74.5	18.2	18.2
	2B	8.2	73.3	18.5	18.7
	3B	8.6	71.2	20.2	19.8
	4B	7.8	75.3	17.0	18.3
	5B	6.6	75.9	17.6	19.3
Class I average		7.7	74.0	18.3	18.9
Class II, S_f 0–20	1B	4.7	68.4	26.9	24.6
	2B	6.9	66.2	26.9	24.9
	3B	6.3	69.6	25.1	25.9
	4B	4.7	69.9	25.4	24.4
	5B	5.5	70.7	23.8	25.5
Class II average		5.6	68.8	25.6	25.0
Class III, HDL–2, 3	1B	6.3	80.1	13.6	40.0
	2B	8.4	79.4	12.1	39.2
	3B	9.3	80.1	10.7	43.2
	4B	6.1	82.3	11.4	41.6
	5B	6.9	81.8	11.4	42.9
Class III Average		7.4	80.8	11.8	41.4

In Table VI the average values obtained in this study are compared with those obtained by Phillips.[33] The general agreement is good. It must be pointed out that Phillips's method of lipoprotein separation is not exactly equivalent to the one followed in this study. Class I in his study is best described as an S_f 12–400 group. His second class is S_f 0–12, and Class III is HDL-1, 2, 3 plus the high-density residue. However, these differences are relatively minor, as the amount of material shifted in each case is less than 10% of the total lipide in each class. Phillips later analyzed the phospholipides found in the serum proteins with a density greater than 1.21 g/ml; these results are shown in Table VI also.

Table VI

Comparison of the average values for the lipoprotein classes obtained in this work with those obtained by Phillips

Lipoprotein class	Work	% of total phospholipides			Number of cases studied
		PE-S	L	S	
I	This work	7.7	74.0	18.3	5
	Phillips	5.7	71.3	23.1	4
II	This work	5.6	68.8	25.6	5
	Phillips	4.4	70.4	25.3	8
III	This work	7.4	80.8	11.8	5
	Phillips	5.2	81.1	13.6	8
$\rho > 1.21$ g/ml	Phillips	6	81	14	6

Every phospholipide appears in every lipoprotein class, but with different distributions in each case. No differences were observed with regard to sex, either by the present author or by Phillips. The significance of the difference between lipoprotein classes is discussed below.

Table VII presents the results of the gas chromatographic analysis of the phospholipide's fatty acids. The gas unit used in this experiment was undergoing modification and additions during the course of the work so that it was impossible to run complete analyses on all samples, and the results, lacking more detailed studies, must be regarded as preliminary. However, certain valid conclusions can be drawn.

The phospholipide fatty acids are not highly unsaturated. Allowing for individual variation, it appears that palmitic and stearic make up more than 50% of the total fatty acids. Palmitic is the primary fatty acid of lecithin and sphingomyelin, while stearic is the primary fatty acid of the phosphatidyl ethanolamine–serine fraction. The primary unsaturated fatty acids are palmitoleic, oleic, linoleic, and arachidonic, with linolenic

almost completely absent. There are as many as ten other components in amounts usually less than 1% that have not been definitely identified at present. However, it is likely that the C^{12}, C^{14}, C^{10} n-chain saturated fatty acids occur in small amounts in all fractions. In the sphingomyelin fraction (see Fig. 7C) there are several slow-moving components following arachidonic that have not been identified. They are not seen in the phosphatidyl ethanolamine-serine or lecithin fractions.

Two total serum phosphatidyl ethanolamine-serine samples were run. There was not enough of this fraction obtained in any of the lipoprotein classes to run fractions separately. Phosphatidyl ethanolamine-serine fractions were pooled for purposes of analysis of the fatty acids after the infrared spectra were obtained on the separate fractions.

The separate runs on the lecithin fractions from the lipoprotein classes indicate that there is no variation in the composition of the fatty acids with respect to the lipoprotein class. A total sphingomyelin fraction and one Class II fraction were run from different individuals. They appear more saturated than the lecithin but less than the phosphatidyl ethanolamine-serine fraction.

Table VII

Principal fatty acids of human serum phospholipides from whole serum and lipoprotein fractions analyzed by gas chromatograph (weight %)

Phospholipide	Subject	Fraction	Fatty acids as % of total fatty acid							
			C^{16}	$C^{16'}$	C^{18}	$C^{18'}$	$C^{18''}$	$C^{18'''}$	$C^{20''''}$	Unidentified
Phosphatidyl ethanolamine-serine	HG	Total	17.9	1.3	35.0	9.4	9.4	0.4	17.9	8.5
	DG	Total	22.1	1.7	37.6	12.1	7.7	-	4.7	13.5
Lecithin	HG	20-400	28.4	0.6	19.7	12.5	22.3	-	11.4	4.4
	HG	0-20	27.4	0.6	23.7	12.3	24.4	-	9.0	2.5
	HG	HDL-2, 3	29.1	0.6	19.5	12.9	24.2	-	10.6	3.1
	HG	Total	28.3	0.6	21.0	12.6	23.6	-	10.3	3.3
	DG	20-400	41.4	2.3	21.1	15.6	11.7	-	-	7.8
	DG	0-20	42.3	2.0	25.4	15.9	8.9	-	2.8	2.8
	DG	HDL-2, 3	46.4	3.6	25.0	14.3	5.4	-	-	5.4
	DG	Total	43.4	2.6	23.8	15.3	8.7	-	0.9	5.3
Sphingomyelin	HG	Total	33.9	0.9	18.3	13.8	21.6	-	6.7	4.6
	DG	0-20	47.0	2.0	20.5	10.8	8.6	1.0	3.2	7.6

DISCUSSION OF RESULTS

The details of the ultracentrifugal characterization of serum lipoproteins have been discussed thoroughly in several publications[52, 54, 65] and are not considered in this study (the reader is referred to these discussions for consideration of problems relating to protein homogeneity and protein purification). Neither are errors in the experimental determination of the phospholipides, which were considered in some detail in a previous publication,[30] discussed further.

The results of Phillips's study[33] agree well with this work in view of the differences between experimental procedures. Each class of lipoproteins shows a distinct phospholipide spectrum. In Phillips's study the distinction between Class I and II is less than that shown in this work. The explanation for this may be found in the ultracentrifugal lipoprotein separation. In Phillips's procedure, taken from the method of Havel et al.,[71] Class I contains lipoprotein with S_f rates above 10 or 12, while S_f 20 was the division point used in this work. The amount of material transferred in this manner, although not large, would be enough to produce the observed differences, as the sphingomyelin concentration is highest in the S_f 0–20 region. It is not altogether impossible, albeit unlikely, for the S_f 10–20 lipoproteins to have a unique phospholipide distribution. There is no evidence for such an assumption, however.

This same type of argument can be applied to the sphingomyelin concentration in Class III. In Phillips's procedure, HDL–1, 2, 3 and the ultracentrifugal residue are included in the third class, while only HDL–2, 3 were analyzed in this work. If HDL–1 approximates Class II, then sphingomyelin will be higher in Phillips's study than in this one. Experimental error may be the primary cause, but this is considered unlikely. These points are somewhat minor, but still bear mentioning.

There is a significant difference between the amount of phosphatidyl ethanolamine–serine reported in Phillips's and in this study. This can be attributed only to a systematic error in one of the procedures used, but it is not possible to ascertain to which work the error should be assigned. The amount of phosphatidyl ethanolamine–serine is small in either case and is subject, therefore, to the largest error. This fraction shows the largest contamination from non-phosphorus-containing materials (see Appendix II). Phillips did not estimate the inherent error in his procedure. It is felt that the phosphatidyl ethanolamine–serine fraction is subject to an error of more than 10% in the method used in this study, however.

Phillips also reported the presence of lysolecithin in all fractions. The average value reported for total serum phospholipides was 7%. The distribution of the lysolecithin was nonuniform; the largest percentage was found in the high-density residue, $\rho > 1.21$ g/ml.[72] In Table VI the Class III values for lecithin attributed to Phillips include 14% lysolecithin. In Classes I and II lysolecithin accounts for no more than 5% of the lecithin value. In the high-density fraction, 60% of the lecithin is lysolecithin.

The presence of lysolecithin in serum has not been demonstrated by any worker other than Phillips at present. All of Phillips's studies were carried out on silicic acid columns, using methanol-chloroform extraction. Until lysolecithin is demonstrated by methods other than these, its existence in the natural state cannot be considered proven. It has been shown that under some conditions chloroform-methanol extraction can hydrolyze phospholipides.[73] And it is considered likely that silicic acid is capable of similar action. Although it is true that these procedures are milder than older methods of lipide extraction, which are known to cause extensive lipide-degradative changes, the possibility that some degradation occurs cannot be entirely eliminated. Although no data have been presented to dispute Phillips's claim, it remains unconfirmed and must be regarded as inconclusive at present.

The distribution of the phospholipides in the lipoprotein classes presents no unusual features, although definite differences exist. Class I contains 20% of the phospholipides in normal serum and has a phospholipide composition not unlike serum. The phosphatidyl ethanolamine-serine fraction is slightly elevated. Class II contains more than 50% of the total serum phospholipides and more than 70% of the total serum sphingomyelin. The phosphatidyl ethanolamine-serine fraction and the lecithin fraction are reduced. Class III contains the lowest concentration of sphingomyelin and the highest concentration of lecithin. The phosphatidyl ethanolamine-serine fraction is similar to Class I. The removal of phospholipide from Class I in the proportions found in Class III produces a composition similar to Class II. This point is discussed further under the section on lipoprotein transformations. Details of the calculation are found in Appendix III.

The values reported by Phillips concerned individuals with average serum lipide levels considerably below those for the cases studied in this work. As there is no significant variation that is not traceable to some other cause, the distribution of serum phospholipides -- both in total serum and in the three classes -- must be unrelated to their concentrations in the serum. This, indeed, is to be expected on the basis of earlier studies of serum lipides in which there was found no variation of the total lipide composition of serum lipoproteins with total serum lipide.[74] In this work alone, individuals with total serum lipide values varying from 13.1 to 8.8 mg/ml were studied and were shown to have similar phospholipide distributions. As all lipides and phospholipides in serum are bound to lipoproteins, and the compositions of lipoproteins are constant regardless of concentration in serum, the phospholipide distribution remains constant also.

In this respect the work of Petersen is of interest.[75] Petersen studied the distribution of total serum phospholipides in starvation. He found a rise in total serum phospholipides that could be accounted for entirely on the basis of a selective rise of the sphingomyelin content of serum. This does not seem possible if the present concepts of lipoproteins as stated above are correct. Petersen's experimental methods, unfortunately, are not considered reliable in view of present knowledge. The rise in total serum phospholipide was not more than 10% of the fasting values, and was

of transient nature. It has been shown that serum lipoprotein levels in S_f 12–400 range increase during long fasting periods (7 days).[76] This produces a general rise in the total serum lipide and phospholipide. A selective rise of one phospholipide would not be expected in this phenomenon, however. Thus, it is likely that the report by Petersen was subject to experimental error.

The phospholipide composition of lipoproteins is of interest in relation to postulated mechanisms of lipide deposition in atherosclerotic plaques. There have been a few reports of phospholipide analyses of aortic plaques.[77,78] The distribution of lipides in aortic plaques is dependent upon the age of the individual plaque and difficult to relate to the initial composition of the material laid down. Hence it would be desirable to have available values of the lipide composition of the aortic plaques as a function of the age of the plaque. It is known that phospholipides of plaques generally have a very high sphingomyelin content. This would seem to preclude the direct incorporation and retention of the serum lipoproteins by aortic plaques, as the concentration of sphingomyelin is never more than 25% for any particular lipoprotein. On the other hand, it is possible that the plaques represent a residue of a lipoprotein fragment. In this connection the lipide composition of newly formed plaques would be of great interest. If the plaques were truly of direct lipoprotein origin, the younger ones should more closely resemble the serum lipoprotein lipide composition.

Judging from the limited data available, the fatty acid composition is roughly the same for the various lipoprotein classes from a given serum. This is consistent with the hypothesis that lipoproteins undergo transformations from one class to another. If the phospholipides in the core of a Class I lipoprotein are eventually found in Class II or III, then the fatty acid distribution must be similar in the different classes. The fact that they are indeed similar does not prove that transformations occur. It could be that all serum phospholipides in a given individual at any one time have the same fatty acid composition regardless of function or location. This latter idea can be related to phospholipide origin and synthesis.

Palmitic acid is the predominant fatty acid of the total phospholipide fraction and also of lecithin and sphingomyelin. The unsaturated acids are mainly oleic and linoleic with some arachidonic. Linolenic was absent, or present only in a minute amount. The amount of palmitoleic is apparently related to the amount of palmitic present.

It is of interest that the primary fatty acid of phosphatidyl ethanolamine is stearic. This acid is rarely reported to be predominant in any compound isolated from humans. In general the degree of saturation found in this study was higher than that reported in the few previous studies,[34,35] but the samples selected were so small that it could be due to individual variation. Further studies are contemplated.

Evidence was obtained for the presence in sphingomyelin of fatty acids having more than twenty carbon atoms (see Fig. 7). This is in agreement with reports on tissue giving a higher percentage of such higher fatty acids in the sphingolipide fraction than in the lecithin or phosphatidyl ethanolamine-serine fraction.[79,80] In this study no attempt was made to identify those compounds. In all fractions analyzed, evidence was obtained for shorter-chain fatty acids in small amounts. The amounts were too small to be accurately measured by using the sizes of samples available. Generally the chromatograms obtained indicated that all normal-chain saturated fatty acids with even numbers of carbon atoms from C^8 to C^{18} or higher are present in serum phospholipides, as are many of their unsaturated analogues. Usually, short-chain components, C^8 to C^{14}, were present in amounts of less than 1% of the total fatty acids.

The distribution of fatty acids in phosphatidyl ethanolamine-serine differed significantly from either the lecithin or sphingomyelin. This fraction was also more highly saturated than the other fractions. Lecithin appeared to be the most unsaturated phospholipide, but still more than 50% saturated. The sphingomyelin fatty acid compostion approximates the lecithin composition.

Luddy et al. reported that the total fatty acid composition of plaque phospholipides was more unsaturated than serum phospholipides, but almost all the unsaturation was in the form of oleic acid with some linoleic and arachidonic.[34] This is the only report in the literature on the fatty acid composition of plaque phospholipides.

THEORETICAL DISCUSSION

In the past few years the accumulation of data on the physical and chemical structures of serum lipoproteins has been proceeding rapidly. This has been stimulated not only by the newly found capability of isolating lipoproteins by ultracentrifugal techniques, but also by the realization that they may be the important entities whose properties are critical in determining the rate of fatty degeneration in arterial walls.[81] In spite of the considerable bulk of such observations and measurements that may be found in the literature, the mechanisms and functions with which lipoproteins are involved are still not clearly understood.[82] In the discussion that follows, an attempt has been made to describe a hypothetical process of lipide dynamics—in the lipoprotein framework—that is consistent with both structural data and the currently accepted concepts of lipide metabolism.

It is necessary for this discussion first to summarize what is known about the lipoproteins in respect to physical and chemical structure. The molecular weights of the lipoproteins are given in Table VIII.

Table VIII

Molecular weights of some serum lipoproteins

Lipoprotein class	Molecular weight	Determination method	Reference
Chylomicron	10^{11}	Volume-density measurements	84
S_f 400	5×10^7	Sedimentation velocity	52
S_f6–8	3×10^6	Sedimentation velocity	52
	3×10^6	Light scattering	85
	6×10^6	Electron microscope	86
HDL–1	1×10^6	Sedimentation velocity	52
HDL–2	3.75×10^5	Sedimentation equilibrium	83
HDL–3	1.75×10^5	Sedimentation equilibrium	83

The smallest well-characterized unit is the HDL–3, with a molecular weight of 175,000 determined by the approach-to-equilibrium method of Archibald.[83] At the other end of the spectrum is the chylomicron, with a molecular weight exceeding 10^{12} units. The chylomicron is not, of course, a true molecule in the sense that there is only one empirical formula, or that well-defined chemical bonds exist between the various subunits; e.g., triglyceride. The chylomicron is better thought of as an oil-in-water dispersion on one hand, while retaining its compositional relationship to the lipoproteins on the other. Between the chylomicron and the HDL–3 lipoprotein there is an almost continuous spectrum of molecular weights.

The S_f 6 molecule has an estimated molecular weight of 3,000,000, the HDL-2 of 400,000, and the S_f 100 of 20,000,000. Figure 12 shows the relationships of molecular weight to protein moiety in the LDL.

Table IX gives the chemical composition of the lipide moiety of the major lipoprotein classes. The data for the S_f 20-400, S_f 0-20, HDL-1, and HDL-2,3 classes are taken from the work of Lindgren et al.[55] and are in agreement with the values of Bragdon et al.[56] The values for the chylomicron are taken from Scanu and Page,[62] who agree with the work of Jobst and Schettler.[87] The high-density residue is approximated from Phillips.[72] The electron microscope indicates that there is a change of shape in the region S_f 10-30, from round spheres at the higher S_f region to objects that appear somewhat like small erythrocytes in the S_f 6-8 region.[92] The HDL molecules are too small to be characterized in the electron microscope with present techniques, although future studies may produce results. Viscosity data on HDL molecules indicate that they are prolate ellipsoids.[83] Figure 13 shows electron micrographs of the principal lipoprotein classes.

Certain pertinent information can be gathered from these tables and figures. Even though the percentage of protein in a chylomicron is small, the large size of the molecule means that the total amount of protein is considerable. Simple removal of triglyceride from chylomicra will not account for the formation of the lower S_f classes, as is true with other components of the chylomicra. These considerations suggest two possibilities: one, the idea of fragmentation of the large chylomicron into many smaller units; or, alternatively, that the chylomicra are unrelated to other serum lipoproteins and are metabolized as a unit.

This latter concept is in conflict with the proposed role of the lipoprotein lipase. The lipoprotein lipase, the principal factor involved in the clearing of lipemic serum, acts only by removing triglyceride from lipoproteins with S_f rates greater than 20 (i.e., having at least 40 to 60% triglyceride by weight).[88,89,55] This enzyme acts in serum by hydrolyzing triglyceride to free fatty acids and mono- or diglycerides. It does not act on phospholipides or cholesterol ester.[90] No enzyme that does act on these compounds has been found in serum, nor is its existence there required to explain any observed lipide transformations. Hence, whereas triglyceride is removed from chylomicra and large S_f molecules, other components are not. Yet no large molecules whose lipide content consists primarily of phospholipide and cholesterol ester have been found in serum, This fact leads one to the idea of fragmentation of the larger units.

Furthermore, there is the idea that removal of large molecules as a unit from serum would be physiologically difficult.[32] Hence a picture begins to emerge. Giant molecules are degraded by (first) the removal of triglyceride by enzymatic hydrolysis and (second) the fragmentation of the residue resulting from the removal of triglyceride to units of smaller molecular weights.

According to the theory proposed by Lindgren et al. chylomicra are formed by emulsification of absorbed triglyceride with HDL molecules.[61]

Table IX

The chemical composition of the lipoprotein classes

Class	Protein (%)	Lipide (%)	Lipide species (in % of total lipide)					Phospholipide (in % of total phospholipide)			Reference
			CSE	CS	TG	PL	UFA	PE-S	L	S	
Chylomicra	2	98	7	3	86	9	-	-	-	-	62, 87
S_f 20–400	13	87	15	8	56	20	2	8	74	18	55, 56
S_f 0–20	25	75	47	12	16	24	1	6	69	26	55, 56
S_f 6–8	25	75	46	11	19	24	1	-	-	-	55
HDL–1	30	70	34	21	19	20	-	-	-	-	61
HDL–2, 3	55	45	24	6	20	44	6	7	81	12	55, 56
$\rho > 1.21$	-	-	-	-	30	70	-	6	81	14	72

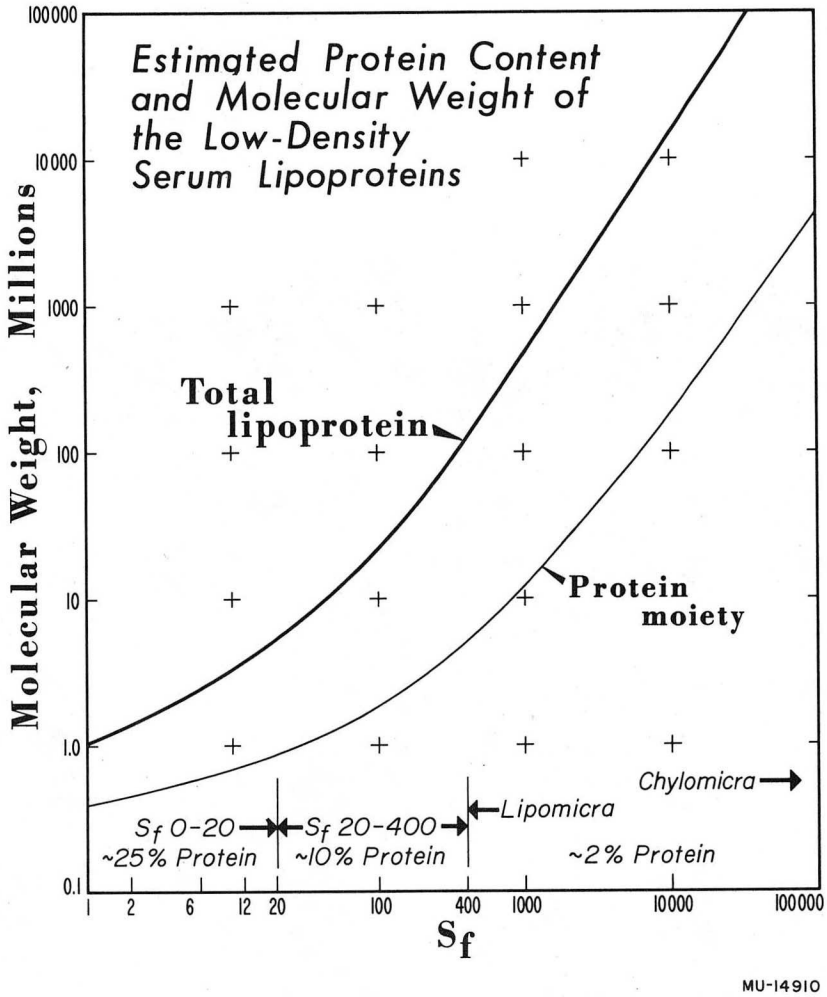

Fig. 12. Total molecular weights of the low-density lipoproteins of S_f 1–10^5 as calculated for spheres (also indicated is the estimated molecular weight of the protein moiety).

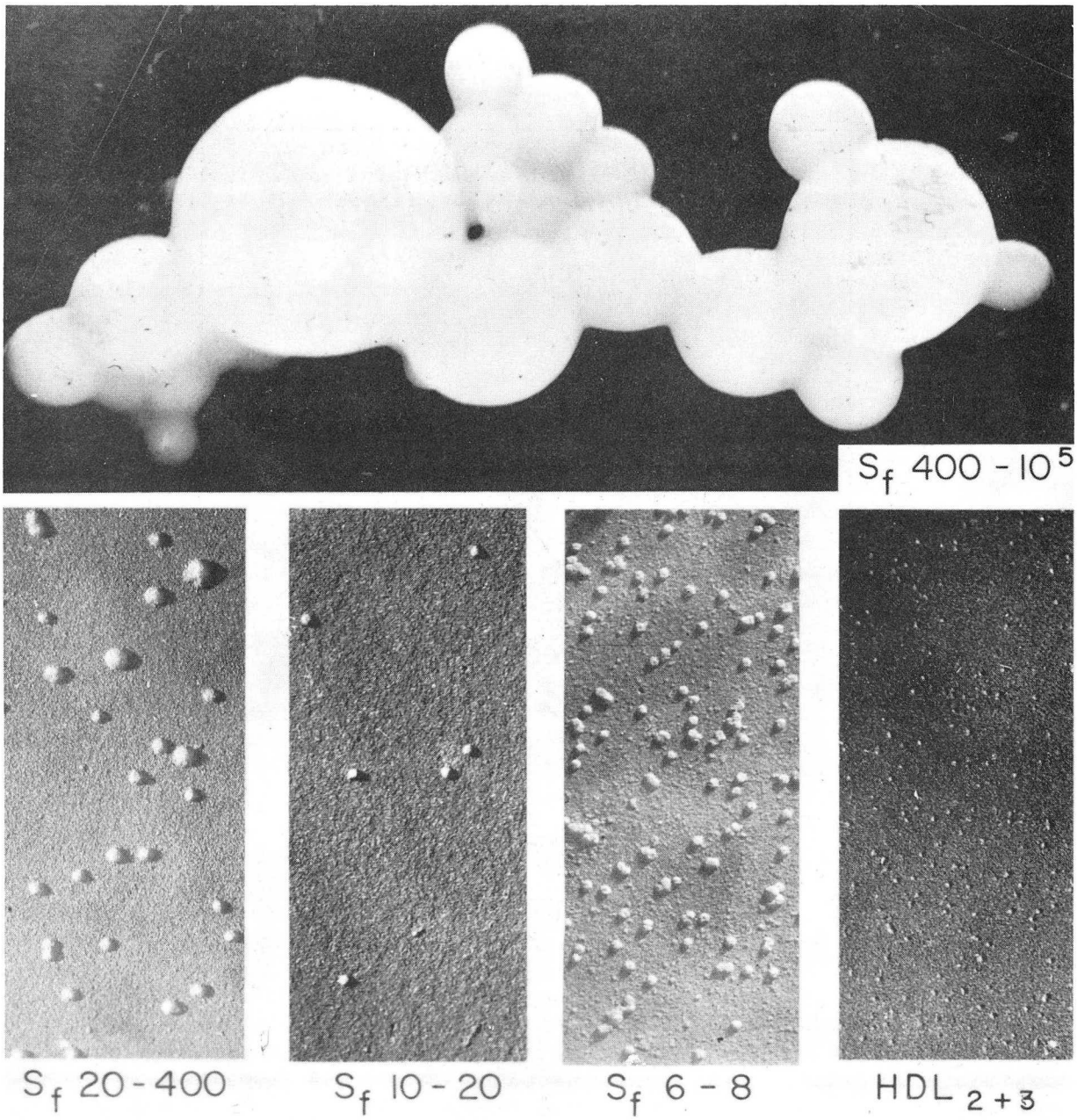

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Fig. 13. Electron micrographs of the major classes of serum lipoproteins. These lipoproteins were isolated ultracentrifugally, fixed in 1% OsO_4, shadowed with Pt-Pd-An alloy, and viewed in a RCA EMU 2-E electron microscope (magnification 36,000 ×).

That HDL molecules per se are the emulsifiers seems at least questionable, in view of recent work by Scanu and Page[62] and of the finding that a chylomicron cannot be reconstituted from HDL and triglyceride that has the same composition as that observed in vivo. The main difficulty involves the protein moiety. If all the protein in the chylomicron is retained in the transformation to smaller molecules--and this seems a reasonable assumption--then it is a simple matter to calculate how many HDL molecules would be formed from the original molecule on the basis of the protein moiety. When this is done (see Appendix III) the calculated composition of the chylomicron does not agree with observations.

Then, of course, it could be supposed that protein from serum is added to the subunits. This hypothesis has no experimental observations to support it and a few that contradict it. Otherwise, it would be an attractive concept indeed. Avigan et al., using radio-labeled amino acids, studied the protein moieties of the lipoproteins synthesized in vivo.[91] They found that the LDL's were labeled much earlier than the HDL fraction, and both were labeled more and earlier than the other serum proteins. The interpretation of this work is open to question and is further discussed below.

Reiterating: Lindgren et al. proposed a model for the LDL of a lipide core surrounded by HDL molecules. Initially the core was largely triglyceride and the HDL were mainly HDL-2, 3. As triglyceride was removed from the core and HDL-2, 3 dissociated from the mantle, the core became predominantly cholesterol ester and phospholipide while the mantle became largely HDL-1. It was not specified how cholesterol or phospholipide was removed from the S_f 0-20 molecules. There could easily be imagined an equilibrium between the HDL and LDL in serum, but this is not borne out, as no correlation between the concentrations of HDL and LDL has been found.[65] However, the homeostatic mechanism controlling the HDL level need not be related to the transformation of the lipoproteins or to the clearing of lipemic serum. Hence, the correlation need not exist.

The data presented earlier on the chemical composition of the lipide moiety, as well as the data on the phospholipides studied in this work, indicate significant differences between the major classes. The phospholipide data are particularly significant in that they show that simple triglyceride removal does not account for the changes in composition of the lipoprotein lipide moiety. Obviously if only triglyceride were being removed the composition of the phospholipides with respect to the individual phospholipides would remain the same.

The C and N terminal amino acid data previously cited also substantiate the differences between the protein moieties of the lipoprotein classes (see Fig. 5).

On the other hand, the relatively sparse data on the fatty acid composition of phospholipides suggest that the distribution of fatty acids in the phospholipide fractions is uniform throughout the lipoprotein spectrum.

Mild organic-phase extraction of purified lipoproteins is of considerable interest with respect to lipoprotein transformations.[43,63,93,94]

The fact that HDL-2, 3 are essentially impervious to the extraction of any of their lipides by ether extraction, while S_f 20-400 loses approximately 60% of the lipide complement, is strongly indicative that a lipide complex not strongly associated with the protein complement exists in the LDL molecules and not in the HDL. The latter, however, do undergo considerable alteration as to density and composition. An HDL solution extracted with ether yields free protein that has a molecular weight of 40,000,[93,94] as well as LDL material. The interpretation of such an unphysiological process remains questionable, aside from the considerations as to the binding of lipide to protein. The fact that the lipide extracted from S_f 20-400 molecules contained appreciable quantities of cholesterol, cholesterol ester, and phospholipide tends to support the idea of a lipide core composed of these substances connected in some manner--possibly Van der Waals forces--to smaller lipoprotein units such as the HDL's.

One of the primary problems related to the whole concept is how the chylomicra are formed. At present the best model is one in which ingested fat is completely hydrolyzed in the intestinal lumen, or hydrolyzed at least to monoglycerides in the case of ingested triglycerides.[32,95-99] After the fatty acids enter the intestinal wall (the mechanism of transport is unknown) they are re-esterified to triglycerides and perhaps some phospholipide and cholesterol ester. The reconstituted fat molecules are combined into chylomicra still within the intestinal wall and then the chylomicra are ejected into the lymph. Only fat in the form of chylomicra enters the circulation.[100,101] In the process of formation of the chylomicra newly synthesized protein is added to the lipide. The cholesterol ester and phospholipide in chylomicra are also newly synthesized, probably in the liver but not necessarily from newly ingested fat. The composition (except for fatty acid types) and hence the mechanism of synthesis of chylomicra is largely independent of the ingested fat.

After the chylomicron is ejected into the lymph, it is transported to the serum, where the lipide clearing factor can act on it.[97] This removes triglyceride and forms the unesterified fatty acids which are picked up by the serum albumin and eventually removed from the circulation by the liver or adipose tissue, depending on the metabolic state of the individual.[101,102,103]

Lipoproteins with S_f rates in the region S_f 20-1000 are not degraded as rapidly as larger molecules, presumably because the action of lipoprotein lipase in hydrolyzing triglycerides is slowed as the amount of triglyceride in the core decreases. The molecules in this region are imagined to be a lipide core consisting largely of triglyceride, but heavily enmeshed with cholesterol esters and triglycerides with many discrete HDL subunits about the core.

The S_f 0-20 class molecule has a similar structure which--because it contains relatively little triglyceride in the core--is not acted upon by lipoprotein lipase. Some other mechanism must be removing the core lipides, which are primarily phospholipide and cholesterol ester. In fact, all triglyceride in the Sf 0-20 molecule is thought to be within HDL-1 molecules that surround the lipide core.

The HDL molecules are also unaffected by lipoprotein lipase and are thought to be similar to the lipoprotein of tissue, with all the lipide enmeshed in the protein in a manner analogous to water absorbed in a sponge.

II

With the above descriptions in mind, it is possible to formulate a unified theory of lipoprotein transformation while remembering that it is only a construct rather than an attempt to describe reality. A lipoprotein need not be a microscopic golf ball representing the lipide core surrounded by smaller balls representing the HDL molecules. Nevertheless such models may be invoked as simplified representations for discussion purposes. Figure 14 is an attempt to pictorialize in a schematic way the sequence of stages in the formation and degradation of serum lipoproteins.

In a sense lipoprotein transformations begin actually before any lipoproteins exist. Ingested fat or at least the fat formed by re-esterification somewhere between the intestinal lumen and the lymph is the starting point. It is probable that in the process of re-esterfication the chylomicron is formed. It is imagined that a newly synthesized protein template exists in the intestinal wall and to this is added newly formed triglyceride along with some cholesterol ester and phospholipide. When the particle has gathered enough material it is ejected into the lymph.

From the lymph, it enters the serum and is acted on by the lipide clearing factor. At this point two actions occur simultaneously. Triglyceride is removed by hydrolysis, the fatty acids being picked up by the serum albumin; protein, phospholipide, and cholesterol ester are removed by formation of HDL-2, 3 molecules which dissociate from the chylomicra. These HDL-2, 3 molecules are not preformed in the liver or intestine but rather form during the process of triglyceride removal from the chylomicra. This is a major point of difference from the Lindgren theory. HDL-2, 3 molecules form sooner and are removed from the chylomicra before HDL-1 molecules.

This process continues at a progressively slower rate until the initial chylomicron is degraded to the S_f 20-30 region. At this point the triglyceride has been largely removed from the lipide core and the formation of HDL-2, 3 molecules no longer occurs. The core is now principally cholesterol ester and phospholipide, with the protein component in the form of HDL-1 molecules. This complex is further degraded by removal of the core lipides, which results in the release of free HDL-1 molecules in the serum. The removal of the S_f 0-20 core lipides cannot be occurring in serum. While the exact mechanism is unknown, it is likely that this process occurs in the liver. It is known that lysophospholipides produce lysis of erythrocytes in the free state in blood. Hence it is unlikely that any hydrolysis of phospholipides would occur in the presence of erythrocytes; and the liver is the most likely place.

Evidence that bears directly on this theory is Avigan et al.'s data on the metabolism of the protein moiety,[91] although based on a somewhat different interpretation from that given by the authors. They observed a rapid initial rise in the activity of the LDL fraction. (Unfortunately they did not separate S_f 0-20 and S_f 20-400 classes). This was followed by a slower and less intense rise in the HDL fraction and a precipitous drop in activity of the LDL fraction. The serum-protein ($\rho > 1.21$ g/ml) background remains essentially constant after a slow initial rise. The authors also found that the LDL was removed from serum more rapidly than HDL, when purified samples

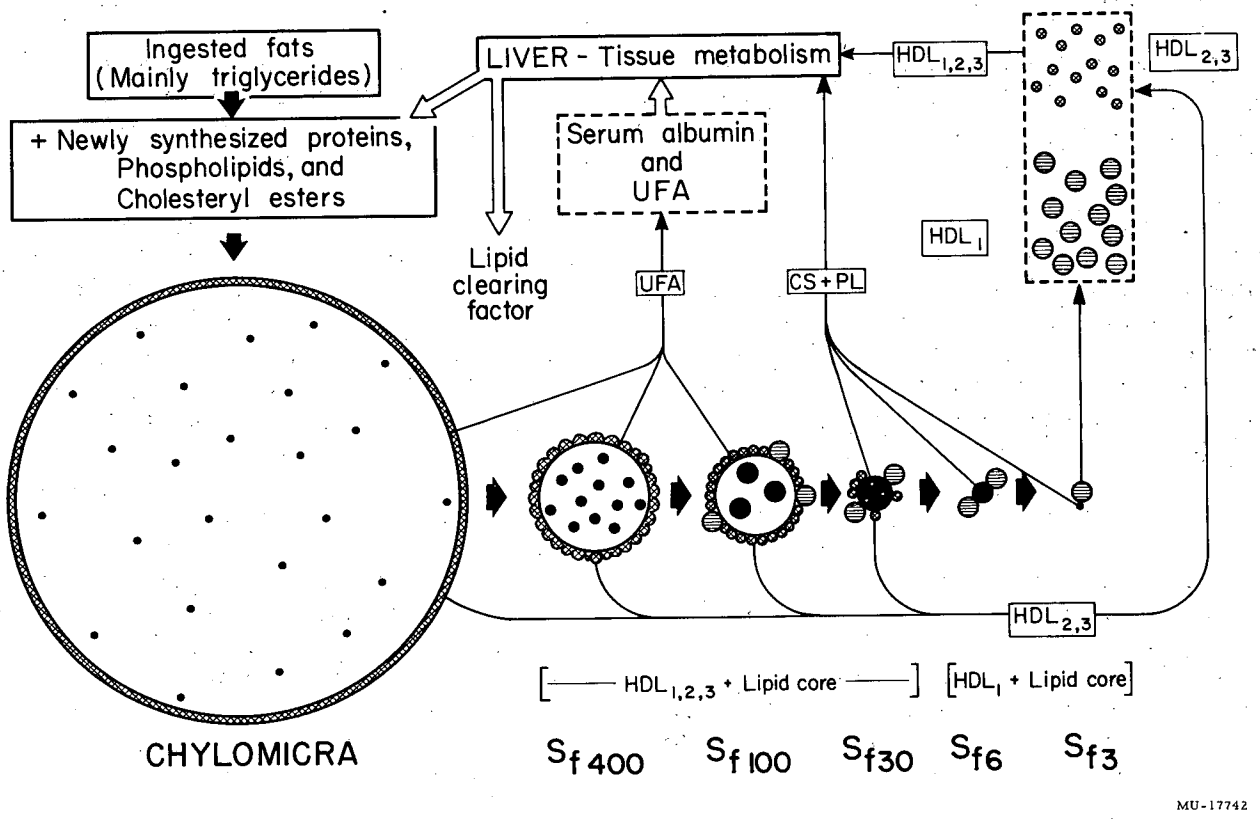

Fig. 14. Proposed model for serum lipoprotein transformations between the various lipoprotein classes found in human sera, presenting schematically the pathway for the transport of ingested fatty acids. See text for further details.

obtained from a donor animal were injected into another fasting animal. In this phase of the experiment no activity was observed in the recipient's HDL or serum residue. The authors inferred from these facts that no interconversion of LDL and HDL was occurring. However, it is possible to interpret the first part of the experiment to indicate that interconversion is occurring; the rapid fall in activity of LDL accounts for the rise in activity of the HDL. In the second part of the experiment, the lipoprotein from a donor may not undergo the same type of transformations in the host, although this must be considered a point against the theory.

The hypothetical removal of HDL phospholipide from the S_f 20–400 molecule is shown in Fig. 15. The calculated values agree quite well with observation. The difficulty arising here is that the amount of protein available is not enough to account for the amount of phospholipide removed from the S_f 20–400 molecule. In this respect it is interesting to reconsider the mild-lipide-extraction work of Hayashi et al.[93] In their extraction of HDL–2, 3 there appeared a lipide-free protein of density 1.32 g/ml. Now, it may be that the HDL–2, 3 molecules, when dissociated from a S_f 20–400 molecule, combine with a lipide-free protein of serum. Thus the particle that is lost from the S_f 20–400 molecule is rich in lipide and relatively poor in protein, and the final composition is determined by addition of lipide-free protein.

The recent work by Scanu and Page[62] and Rodbell et al.[64] has proven beyond doubt that the same type of protein exists in HDL–2, 3 molecules as in chylomicra, and also that there exist more than one protein species in chylomicra but only one in HDL–2, 3. These determinations were based on the fingerprint method of characterization of protein species. Rodbell et al. were able to show the identity of HDL–2, 3 protein with one of three proteins they found in chylomicra. Scanu and Page reported that chylomicra proteins were similar to a protein obtained in pseudo-chylomicra formed by incubating serum with coconut oil emulsions. There have not been, at present, any reports in the literature of fingerprint studies on S_f 20–400 or S_f 0–20 protein moieties.

Considerable further experimental work would be necessary to confirm or deny this theory.

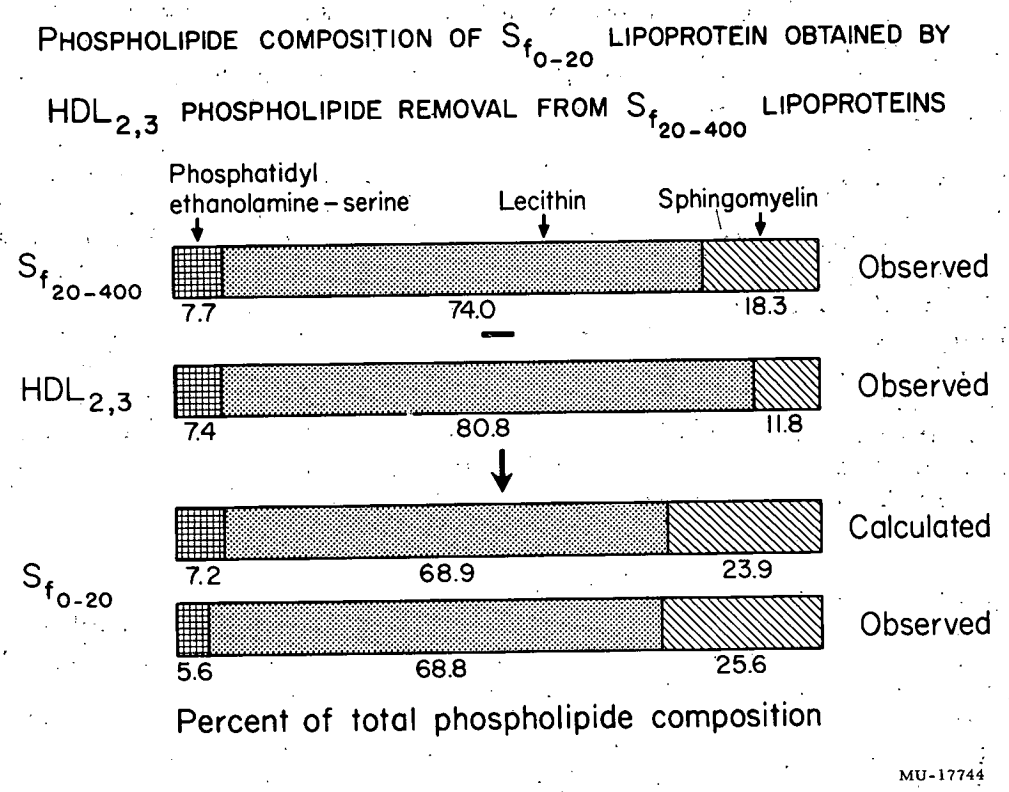

Fig. 15. Observed results of phospholipide removal from the S_f 20–400 molecule in ratio present in HDL 2–3 molecules compared with the phospholipide composition of the S_f0–20 molecules. Details of this calculation are given in Appendix III.

CONCLUSIONS

This work, coupled with the published results of Phillips, has established the average normal values of the main types of phospholipides in human serum with respect to the major lipoprotein classes, along with the expected ranges of values. It will now be possible to study pathological conditions to determine whether there are abnormalities in phospholipide values specifically associated with a particular disease.

With respect to the composition and structure of the various lipoprotein classes, it has been found that the ratios of phospholipide types differ significantly from one class to another. The most striking variation is in the sphingomyelin content, for which the average percentage (of total phospholipides) is 18.3% in S_f 20–400, 25.6% in S_f 0–20, and 11.8% in HDL-2, 3. Thus the largest fraction of the sphingomyelin in serum is carried by S_f 0–20 lipoproteins, since these are in general the most abundant class. Limited study of the fatty acid compositions of the phospholipides suggests that the common long-chain fatty acids--palmitic, stearic, oleic, linoleic, and arachidonic--are present in all, with the saturated acids predominating. Sphingomyelin contains, in addition, significant amounts of fatty acids not found in lecithin or phosphatidyl ethanolamine. These fatty acids probably contain more than twenty carbon atoms, and possibly hydroxyl groups, such as the fatty acids known to exist in tissue sphingomyelins. Some possible relationships of these experimental findings to atherosclerosis, phospholipide function, and lipoprotein transformations may be inferred, and the latter in particular has been treated at some length.

Since none of the lipoprotein classes corresponds in either lipide or phospholipide composition to that of atheromatous plaques, it is difficult to establish a direct association. The increase in sphingomyelin content of plaques with age suggests that this component may have special importance in their development. The analytical methods developed and described here should be applicable in further investigation of such possible relationships.

The role of phospholipides in lipoproteins and human metabolism still needs elucidating. It has been assumed that they function chiefly as emulsifying and structural agents both in lipoproteins and in cellular morphology.[104] They are believed to provide the mode of linkage between proteins and neutral lipides. There is evidence that they do not act primarily as vehicles of fatty acid transport. The experimental results obtained in this work--i.e., the ratios of the various phospholipides in serum and in the lipoproteins--do not make any appreciable contribution to these basic concepts. Hence the normal metabolic function assigned to phospholipides in serum must remain that of an emulsifying and stabilizing agent for fatty substances.

The phospholipide compositions do have a bearing on the interrelationships between lipoproteins and their possible transformations in the metabolic process. The differences that have been established between the major lipoprotein classes are sufficient to rule out the possibility that the phospholipides of one class can be the result of intact transfer from another class. A modified theory of lipoprotein transformation has been presented, taking into account this and other relevant observations that have been reported recently.

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APPENDIX

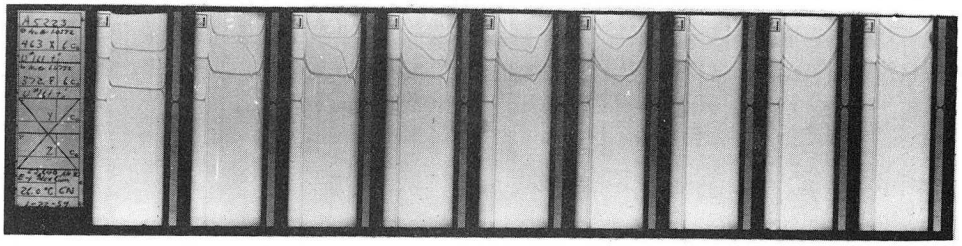
I. Flotation Patterns

This appendix presents the complete analytical ultracentrifugal flotation patterns of the lipoprotein fraction for the five subjects studied in this work. The S_f 20–400 and S_f 0–20 lipoproteins were floated in a medium of density 1.063 g/ml by the addition of NaBr. The HDL patterns were obtained in a medium of 1.21 g/ml by addition of NaBr. All runs were made at a rotor speed of 52,640 rpm at 26°C in double-sector ultracentrifuge cells. The reference compartment contained a solution of pure NaBr in distilled H_2O of the same density as the sample solution. The time of each exposure is given along the top of the S_f 20–400 strip and is in minutes after the centrifuge obtained maximum velocity. Every so often, technical difficulties in the operation of the camera or in development resulted in the loss of an exposure.

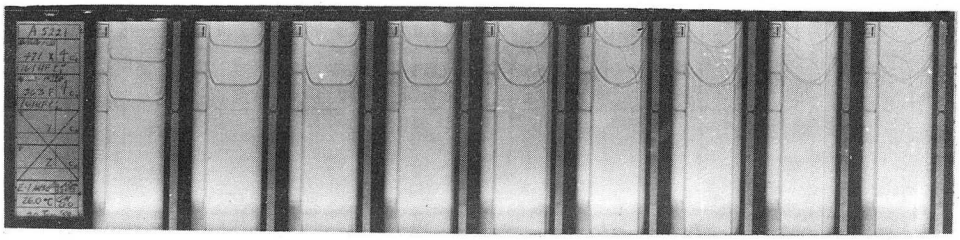
In all film strips the upper pattern is the lipoprotein fraction that was analyzed in this study. The second pattern in the S_f 20–400, S_f 0–20, and HDL–1, 2, 3 strips is the second ml from the preparative run; it was included as a check on the separation of the lipoprotein fractions. In the HDL–2, 3 strip four patterns are usually presented, representing the complete contents of the preparatory tube. Frequently some HDL–1 remained in the final HDL–2, 3 preparation, but in an amount reduced from that initially present. The first frame on each strip is the identification card designating sample and operating conditions. For further details see text or References 52, 66. Patterns are shown in Figs. 16-20.

Time, min, U.T.S.

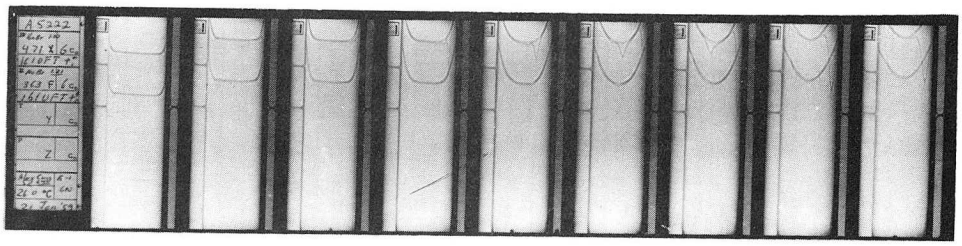
-2 0 2 6 14 22 30 48 64



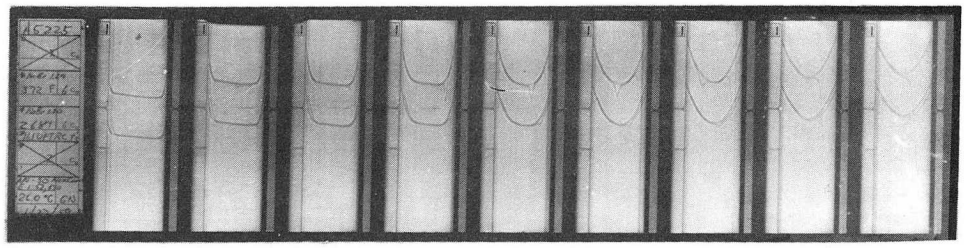
$S_{f_{20-400}}$



$S_{f_{0-20}}$



$HDL_{1,2,3}$



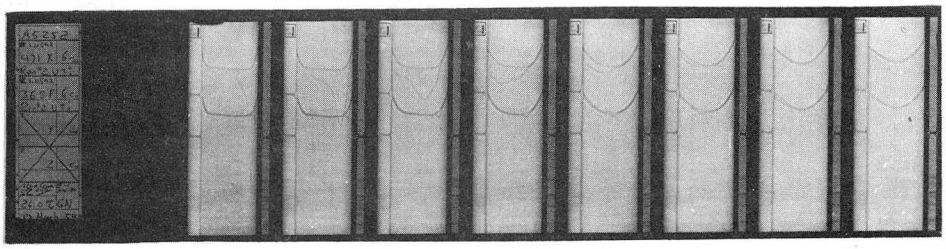
$HDL_{2,3}$

Subject L.M.

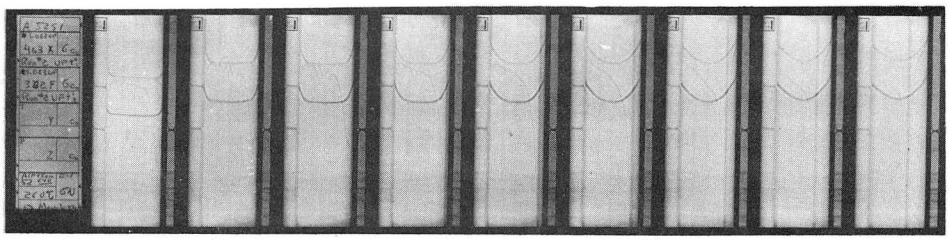
ZN-2332

Time, min, U.T.S.

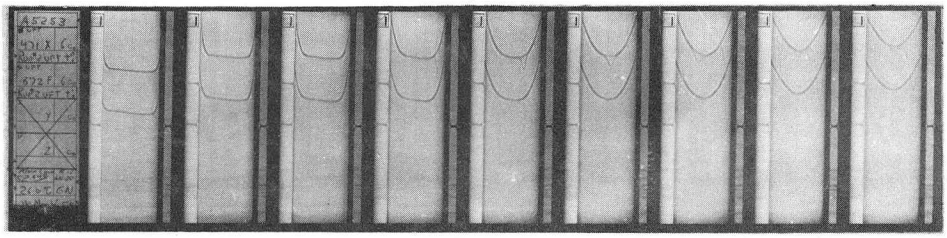
-2 0 2 6 14 22 30 48 64



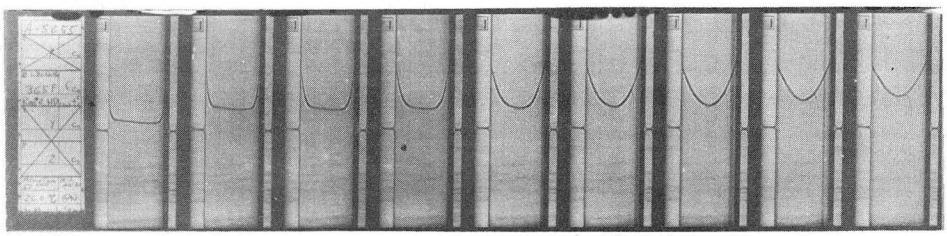
$S_{f_{20-400}}$



$S_{f_{0-20}}$



$HDL_{1,2,3}$



$HDL_{2,3}$

Subject F.D.

ZN-2330

Time, min, U.T.S.

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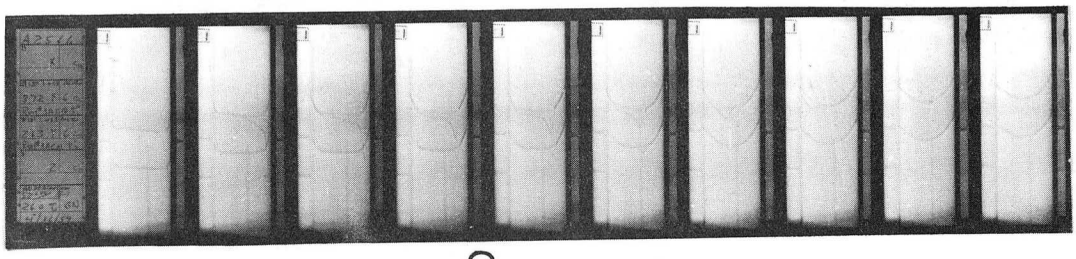
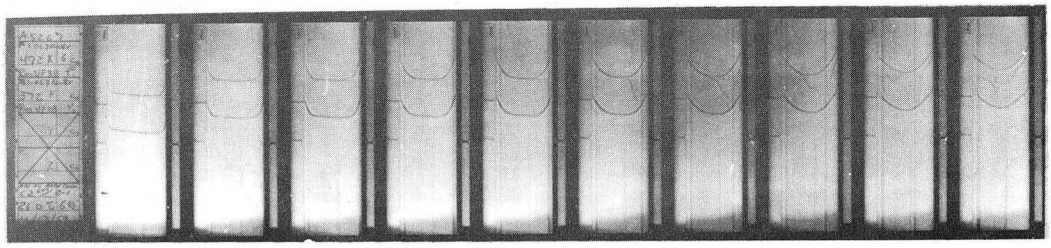
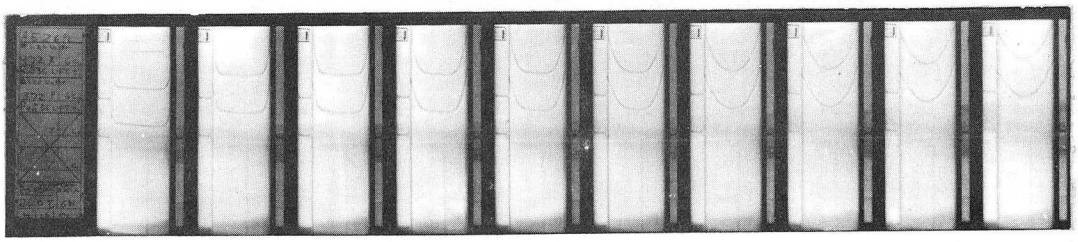


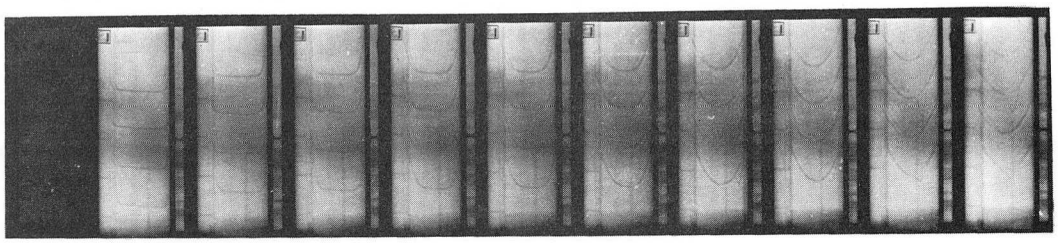
$S_{f_{20-400}}$



$S_{f_{0-20}}$



$HDL_{1,2,3}$



$HDL_{2,3}$

Subject H.G.

ZN-2333

Time, min, U.T.S.

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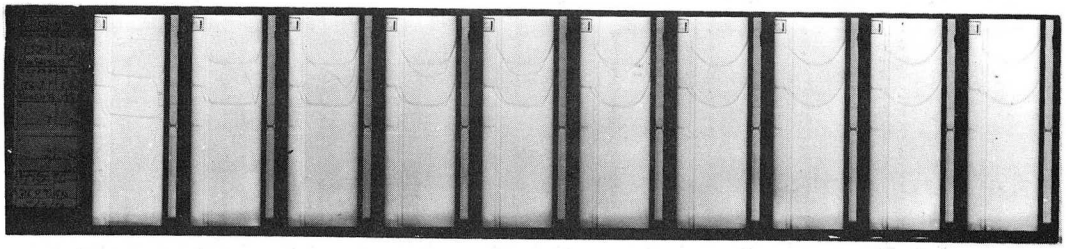
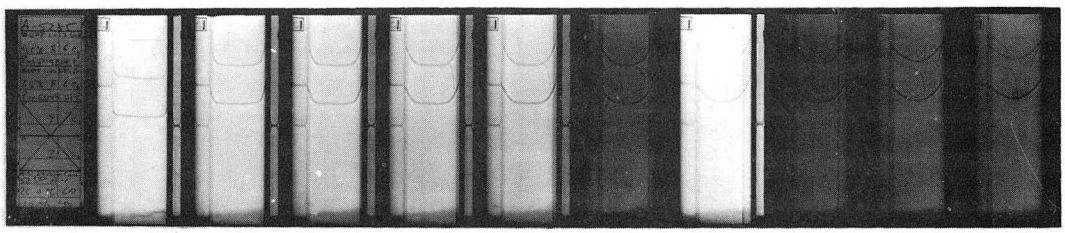
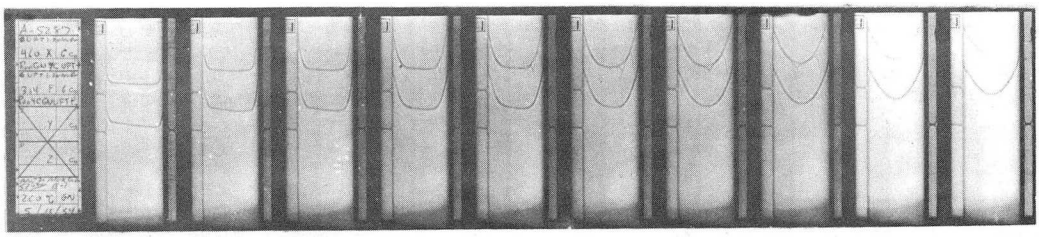


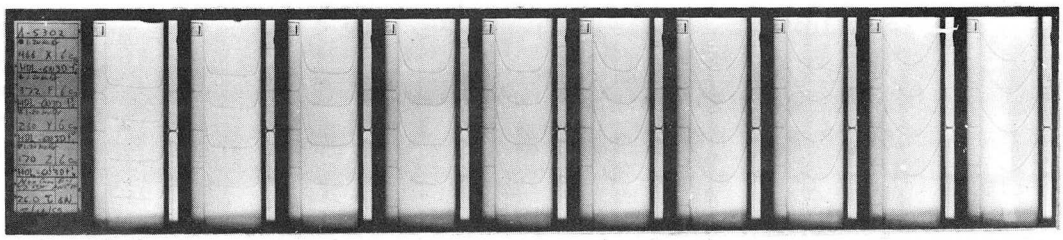
$S_{f_{20-400}}$



$S_{f_{0-20}}$



$HDL_{1,2,3}$



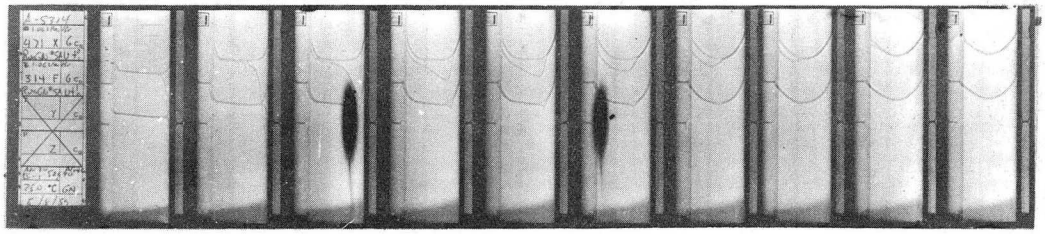
$HDL_{2,3}$

Subject D.G.

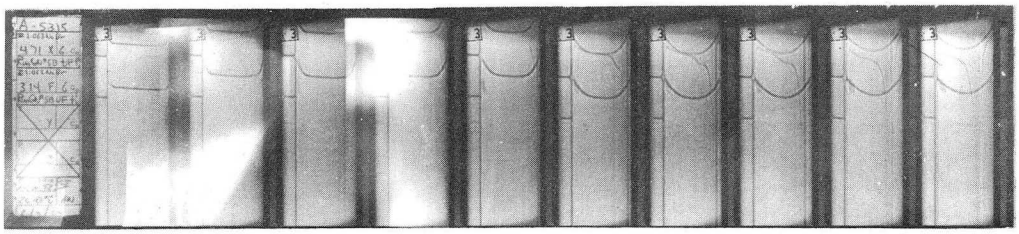
ZN-2334

Time, min, U.T.S.

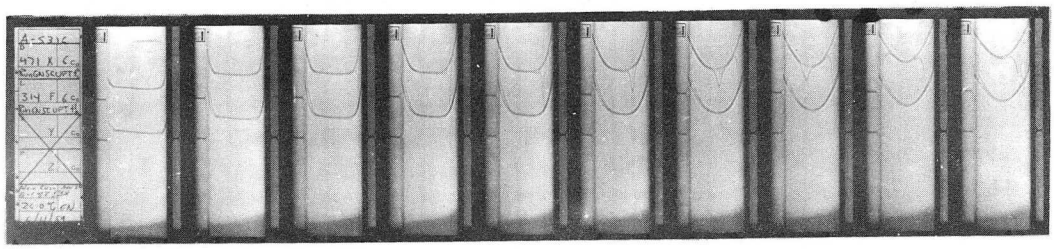
-2 0 2 6 8 14 22 30 48 64



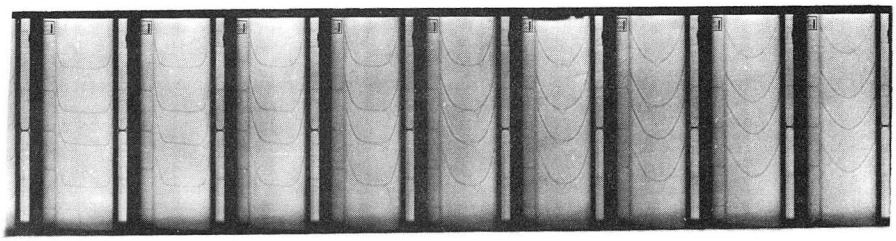
$S_{f_{20-400}}$



$S_{f_{0-20}}$



$HDL_{1,2,3}$



$HDL_{2,3}$

Subject R.G.

ZN-2331

II. Tables of Results

The tables presented in this appendix give the actual results of the chromatography of each total lipide extract obtained from the lipoprotein fractions. The gravimetric, infrared, chemical data are presented on each fraction. The chemical values were obtained by multiplying the chemical phosphorus in mg by the factor 25. The phosphorus determinations were made by the method of Griswold, Humoller, and McIntyre.[69] Infrared values were determined as described in the text. Before weighing, each fraction was desiccated in vacuum. Chromatographic recovery was based on gravimetric weight in each case.

Table X

Comparison of gravimetric, chemical, and infrared determinations on fractions obtained from chromatography of phospholipides from lipoprotein fractions of L. M.

Fraction No. S_f 20–400	Weights of Fractions (in mg) Infrared	Chemical	Grav. Wt.	Recovery 99.7%
I	-	-	88.20	
II	-	0.04	1.67	
III	1.59	1.50	1.75	
IV	14.60	14.00	14.81	
V	5.17	8.95	6.00	
Totals III, IV, V	21.36	24.45	21.96	$\Delta E \frac{Chem}{IR} = 14.5\%$
Fraction No. S_f 0–20				Recovery 101.0%
I	-	-	62.23	
II	-	0.04	0.88	
III	1.00	1.15	2.44	
IV	12.40	12.90	12.53	
V	7.49	6.35	9.32	
Totals III, IV, V	20.89	20.40	24.29	$\Delta E \frac{Chem}{IR} = -2.0\%$
Fraction No. HDL–2-3				Recovery 95.6%
I	-	-	28.48	
II	-	0.08	1.52	
III	1.40	1.38	1.70	
IV	15.10	16.40	15.72	
V	5.57	6.90	6.43	
Totals III, IV, V	22.07	24.68	23.85	$\Delta E \frac{Chem}{IR} = 11.8\%$

% PL of TLE in serum by Chem P is 28.8

Table XI

Comparison of gravimetric, chemical, and infrared determinations on fractions obtained from chromatography of phospholipides from lipoprotein fractions of F. D.

Fraction No.	Weights of Fractions (in mg)			
S_f 20–400	Infrared	Chemical	Grav. Wt.	Recovery 98.8%
I	-	-	110.49	
II	-	0.06	2.06	
III	2.17	1.86	2.84	
IV	17.23	16.20	18.22	
V	6.91	6.95	7.27	
Totals III, IV, V	26.31	25.01	28.33	$\Delta E \frac{Chem}{IR} = -5.0\%$
Fraction No. S_f0–20				Recovery 99.2%
I	-	-	81.00	
II	-	0.03	2.98	
III	2.03	2.02	3.17	
IV	19.05	18.70	21.15	
V	8.15	7.15	9.11	
Totals III, IV, V	29.23	27.87	33.43	$\Delta E \frac{Chem}{IR} = -4.6\%$
Fraction No. HDL–2-3				Recovery 101.4%
I	-	-	31.17	
II	-	0.06	1.30	
III	1.90	1.56	2.38	
IV	16.06	15.20	17.11	
V	4.51	4.31	5.44	
Totals III, IV, V	22.47	21.07	24.93	$\Delta E \frac{Chem}{IR} = -6.2\%$

%PL of TLE in serum by Chem P is 30.0

Table XII

Comparison of gravimetric, chemical, and infrared determinations on fractions obtained from chromatography of phospholipides from lipoprotein fractions of H. G.

Fraction No.	Weights of Fractions (in mg)			
S_f 20–400	Infrared	Chemical	Grav. Wt.	Recovery 99.1 %
I	-	-	95.84	
II	-	0.04	1.41	
III	2.13	1.78	2.97	
IV	16.82	17.04	18.01	
V	5.73	6.30	6.59	
Totals III, IV, V	24.68	25.12	27.57	$\Delta E \frac{Chem}{IR} = 1.9\%$
Fraction No. S_f 0–20				Recovery 100.5 %
I	-	-	70.58	
II	-	0.02	1.41	
III	1.67	1.53	2.64	
IV	18.28	17.71	20.22	
V	6.56	7.55	7.47	
Totals III, IV, V	26.51	26.79	30.33	$\Delta E \frac{Chem}{IR} = 1.2\%$
Fraction No. HDL–2-3				Recovery 103.3%
I	-	-	30.09	
II	-	0.05	2.02	
III	2.57	2.31	4.14	
IV	20.49	20.65	22.51	
V	4.67	6.80	7.34	
Totals III, IV, V	27.73	29.76	33.99	$\Delta E \frac{Chem}{IR} = 7.2\%$

% PL of TLE in serum by Chem P is 33.4

Table XIII

Comparison of gravimetric, chemical, and infrared determinations on fractions obtained from chromatography of phospholipides from lipoprotein fractions of D. G.

Fraction No.	Weights of Fractions (in mg)			
S_f 20–400	Infrared	Chemical	Grav. Wt.	Recovery 94.0%
I	-	-	80.91	
II	-	0.04	2.13	
III	1.49	1.28	2.24	
IV	14.28	15.00	15.17	
V	3.34	4.43	4.02	
Totals III, IV, V	19.11	20.71	21.43	$\Delta E \frac{\text{Chem}}{\text{IR}} = 8.4\%$
Fraction No. S_f 0–20				Recovery 99.4%
I	-	-	78.38	
II	-	0.05	1.61	
III	1.28	1.48	2.45	
IV	19.37	18.20	21.13	
V	6.45	8.24	7.74	
Totals III, IV, V	27.10	27.92	31.32	$\Delta E \frac{\text{Chem}}{\text{IR}} = 3.1\%$
Fraction No. HDL–2-3				Recovery 102.5%
I	-	-	44.14	
II	-	0.14	2.21	
III	3.07	3.52	4.19	
IV	27.77	28.20	29.79	
V	4.48	4.93	4.98	
Totals III, IV, V	35.32	36.65	38.96	$\Delta E \frac{\text{Chem}}{\text{IR}} = 3.8\%$

%PL of TLE in serum by Chem P is 26.3

Table XIV

Comparison of gravimetric, chemical, and infrared determinations on fractions obtained from chromatography of phospholipides from lipoprotein fractions of R. G.

Fraction No. S_f 20–400	Weights of Fractions (in mg) Infrared	Chemical	Grav. Wt.	Recovery 99.4%
I	-	-	91.85	
II	-	0.07	2.24	
III	1.51	1.49	2.60	
IV	17.31	16.50	18.22	
V	4.24	4.70	4.78	
Totals III, IV, V	23.06	22.69	25.60	$\Delta E \frac{Chem}{IR} = 1.6\%$
Fraction No. S_f 0 – 20				Recovery 101.5 %
I	-	-	79.28	
II	-	0.03	1.59	
III	1.61	1.65	2.60	
IV	20.70	23.00	24.63	
V	5.96	6.47	6.82	
Totals III, IV, V	28.27	31.12	34.05	$\Delta E \frac{Chem}{IR} = -8.9\%$
Fraction No. HDL–2-3				Recovery 102.5 %
I	-	-	26.37	
II	-	0.05	1.30	
III	1.60	1.61	2.31	
IV	18.77	18.30	21.08	
V	2.98	3.35	3.42	
Totals III, IV, V	23.35	23.26	26.81	$\Delta E \frac{Chem}{IR} = 0.5\ \%$

% PL of TLE in serum by Chem P is 26.9%

III. Miscellaneous Computations

Calculation of Molecular Weight of Chylomicron

For purposes of this calculation the following assumptions were made: (a) the diameter of the chylomicron is 1μ, which is an average value of serum chylomicra, and (b) the density of the chylomicron is that of a triglyceride; i.e., tripalmitin. The calculation from here on is straightforward.

Diameter of chylomicron	1 μ, 10^{-4} cm
Density, ρ, of chylomicron (tripalmitin)	0.88 g/cc
Volume of chylomicron ($=4/3\pi r^3$)	5.24×10^{-13} cc
Mass of chylomicron ($V \times \rho$)	4.61×10^{-13} g
Avogadro's number	6.02×10^{23}

The molecular weight of a chylomicron is given by Avogadro's number times the mass:

$$(6.02 \times 10^{23}) \times (4.61 \times 10^{-13}) = 2.8 \times 10^{11} \text{ molecular}$$

weight units.

Reconstruction of Chylomicron from HDL-2, 3

Composition of chylomicron in per cent of total mass:[62, 93]

Protein	CSE	CS	TG	PL	M. W.
2	2	3	85	8	10^{11}

Molecular weight of protein moiety of chylomicron. . . . 2×10^9

Molecular weight of protein moiety of HDL-2, 3. . . . . 10^5

Number of HDL-2, 3 molecules needed to supply protein to chylomicron. . . . . 2×10^4

HDL-2, 3 composition in per cent of total mass:[55, 56]

Protein	CSE	CS	TG	PL	M. W.
54	12	2	10	22	2×10^5

When these values are multiplied by the number of molecules needed to supply protein to the chylomicron and the molecular weight of HDL-2, 3, the following values are obtained:

Protein	CSE	CS	TG	PL
2×10^9	4.8×10^8	8×10^7	--	9×10^8

Compared with the experimentally observed values in chylomicra,

2×10^9,	2×10^9,	3×10^9	--	8×10^9,

The triglyceride values are not relevant.

It is seen that for every lipide component the values are too low by a factor of 10 or more. It is likely, therefore, that cholesterol, cholesterol ester, and phospholipides are incorporated into the chylomicron, as well as triglyceride, in a manner other than simple emulsification of HDL-2, 3 molecules. This was observed experimentally also by Scanu and Page,[62] who tried to reconstruct chylomicra in vitro from whole serum and coconut oil emulsions. The amount of protein picked up by the pseudo-chylomicron was in agreement with that found in the natural product, but phospholipide and cholesterol ester values fell below those reported for the natural product.

Removal of HDL-2, 3 Phospholipides from S_f 20-400 Molecules

The average molecule weight for the S_f 20-400 lipoprotein is 2×10^7 units, of which 3.3×10^6 is phospholipide. The average molecular weight of an HDL-2, 3 lipoprotein molecule is 10^5 units, of which 4.2×10^4 is phospholipides. For the S_f 0-20 lipoproteins, 6×10^5 units is phospholipides.

It is obvious that many HDL-2, 3 molecules could be formed from one S_f 20-400 molecule. However, the number of S_f 0-20 molecules is severely limited. If the conversion of S_f 20-400 to S_f 0-20 is one to one, and all phospholipides are conserved in the HDL-2, 3 and S_f 0-20 molecules, then 600, 000 units of the phospholipide moiety of S_f 20-400 is in S_f0-20 lipoproteins and 2, 700, 000 units in the HDL-2, 3 lipoproteins. This calculation is shown below. The numbers represent the molecular weights of individual phospholipide moieties in the lipoproteins.

	PE-S	L	S	Total
S_f 20-400	250, 000	2, 440, 000	610, 000	3, 300, 000
HDL-2, 3	200, 000	2, 180, 000	320, 000	2, 700, 000
Difference available for				
S_f 0-20	50, 000	260, 000	290, 000	600, 000
In % of total	5	42	43	100
Observed % in S_f 0-20	6	69	25	100

This obviously does not agree with observation. It was found that converting one S_f 20-400 lipoprotein to two S_f 0-20 molecules would not produce agreement, either. However, if the conversion is one S_f 20-400 molecule going to three S_f 0-20 lipoprotein molecules and many HDL-2, 3 molecules, the results are as given below.

	PE-S	L	S	Total
S_f 20-400	250, 000	2, 440, 000	610, 000	3, 300, 000
HDL 2, 3	120, 000	1, 200, 000	180, 000	1, 500, 000
Difference available for				
S_f 0-20	130, 000	1, 240, 000	430, 000	1, 800, 000
In % of total	7.2	68.9	23.9	100
Observed % in S_f 0-20	5.6	68.8	25.6	100

Three S_f 0–20 molecules would contain a protein moiety of 2,400,000, which is an amount of protein almost equal to that found in the S_f 20–400 molecule, and does not leave much, if any, for the HDL–2, 3 molecules. This point is discussed in the text.

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Rosemary Priest
Information Division

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