Lipid Composition of Lipoproteins of Normal Human Plasma*

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It is now established that human blood plasma contains at least three distinct groups of lipoproteins. Although there are several physical methods of isolating lipoproteins, ultracentrifugation has proved to be the most precise. By this means, lipoprotein groups of known mean hydrated density have been isolated and characterized. These differ from each other in peptide composition (1, 2), lipid content (3, 4, 5), turnover time (6), and response to various pathological conditions (7, 8). However, their relationships to each other and their function in the transport of lipids are not yet understood. A more detailed knowledge of the composition of each group might help in resolving these questions.

In recent years, much work has been done on methods of analysis of mixtures of long-chain fatty acids. Such methods include column, paper and gas-liquid chromatography, enzymic oxidation, and alkali isomerization. These methods have been applied to the analysis of the fatty acids of whole plasma, plasma phosphatides, cholesterol esters, triglycerides (9–13), and plasma lipoproteins (14, 15).

It may reasonably be assumed that phosphatides and cholesterol esters play an essential part in maintaining the structure of lipoproteins since they are found in all lipoprotein groups so far examined (with the possible exception of a very high density lipoprotein (16)). However, their relative importance may vary from one lipoprotein type to another. For instance, phosphatides may be much more important in stabilizing chylomicrons and low density lipoproteins than they are in stabilizing the peptide-rich, high density lipoproteins. Avigan (17) has presented evidence that the ways in which phosphatides and cholesterol are bound in these two classes of lipoproteins do differ. Such differences could be related to the fatty acid composition of the lipids since the introduction, for example, of cis double bonds into a fatty acid chain alters the shape and physical properties of the molecule (18).

Investigations of the problem of atherosclerosis led to the discovery of a relationship between hypercholesterolemia and the elevation of specific lipoprotein classes. Recent work has established that ingestion of unsaturated fatty acids leads to the elevation of specific lipoprotein classes. Recent work has also demonstrated that ingestion of unsaturated fatty acids leads to the elevation of specific lipoprotein classes. Recent work has established that ingestion of unsaturated fatty acids leads to the elevation of specific lipoprotein classes. Recent work has established that ingestion of unsaturated fatty acids leads to the elevation of specific lipoprotein classes. Recent work has established that ingestion of unsaturated fatty acids leads to the elevation of specific lipoprotein classes. Recent work has established that ingestion of unsaturated fatty acids leads to the elevation of specific lipoprotein classes.

We have thus examined the fatty acid composition of three well-defined lipoprotein fractions and, to some extent, the fatty acid composition of their phosphatides, cholesterol esters, and triglycerides.

EXPERIMENTAL PROCEDURE

Methods

Lipid extraction was performed by the method of Folch et al. (21). Peptide was determined by filtering the extraction mixture through a tared, sintered glass funnel and washing the chloroform-methanol insoluble residue with 70% (volume for volume) aqueous ethanol and then with boiling distilled water. The funnel and contents were dried in an oven at 105°C and later in a vacuum to constant weight. Lipid was determined by taking aliquots of the washed extract, evaporating the solvent under a heat lamp, and drying in a vacuum to constant weight.

Total phosphorus was determined by the method of King (22). Phosphatide was estimated by multiplying this value by the factor 25. Total sterol was determined by the method of Zlatkis et al. (23). Results were expressed in terms of cholesterol. Since it has been reported that unsaturated fatty acids interfere with this method (24), samples of lipids of each lipoprotein group were assayed simultaneously by this method and that of Sperry and Webb (25). The results agreed to within 5%.

Iodine number was determined by the method of Yasuda (26). This method was found to give more consistent results than that of Wijes (27), although some low values are obtained with polyene fatty acids. Total fatty acids were estimated by saponifying a suitable weight of lipid in 0.1 N alcoholic potassium hydroxide and back-titrating to the phenolphthalein end point with 0.1 N HCl. In calculating the percentage of fatty acids, a mean molecular weight of 283 was assumed. Polyunsaturated fatty acids were determined by a modification of the method of Herb and Riemenschneider (28). The monoenoic fatty acid content expressed as oleic acid was estimated from the iodine number by subtracting the contribution of the polyunsaturated fatty acids and cholesterol. Saturated fatty acids were estimated by subtracting the total unsaturated acid content from the total fatty acid content.

Preparation of Lipoprotein Fractions—The lipoproteins studied include β-lipoproteins in the density range from 0.96 to 1.00 (S, 400 to 20), β-lipoproteins of density 1.03 (S, 10 to 2), and...
the α-lipoproteins in the density range from 1.09 to 1.14. All S, values are for solvent density 1.063 at 25°C.

Each batch of lipoproteins was prepared from resin-collected plasma or serum (29) from a single human volunteer. Two samples of β-lipoproteins of density 1.03 were prepared from Fraction III, method 10B (30), from pooled serum. The results from these samples were within the range found for plasma from single subjects. All manipulations involved in the preparation of the lipoproteins were carried out in a cold room maintained at 0°C and all solutions used contained 0.1 g per liter of the disodium salt of ethylenediaminetetraacetic acid (5).

The first stage in the preparation was the flotation of the β-lipoproteins. A sufficient volume of a solution containing sodium chloride (2.90 M) and potassium bromide (2.90 M) was added to the plasma to bring the density to 1.063. This was then centrifuged at 105,000 × g for 18 hours and the β-lipoprotein concentrate was removed from the top of the tube. The density of the lower phase was adjusted to 1.21 by dialysis against a calculated volume of sodium chloride-potassium bromide solution, 1.82 M with respect to each salt. It was centrifuged at 105,000 × g for 18 hours and then the α-lipoprotein concentrate was removed from the top of the tube.

The β-lipoproteins were further fractionated in a density gradient tube (5, 31) to give lipoproteins of density less than 1.00 and density 1.02 to 1.04. The less dense lipoprotein solution was dialyzed for 48 hours against several changes of 0.15 M sodium chloride and centrifuged at 20,000 × g in a Spinco model L ultracentrifuge (rotor S.W. 39L) for 60 minutes. The chylomicrons which formed a creamy layer at the top of the tube were removed and the transparent lower phase, lipoproteins of density 0.96 to 1.00, collected.

Preparation of Lipid Components—Phosphatides were precipitated from a solution of the lipid in light petroleum (30°C–60°C). A small amount of saturated alcoholic MgCl2 was used to facilitate the precipitation. The precipitate was extracted with chloroform and the phosphatide precipitated at least once without adding MgCl2. As a check on this method the phosphatides were removed from the lipids of one lipoprotein preparation of density 1.03 by adsorption on Florisil (32). The fatty acid content of this fraction was then calculated by difference from analyses of the original lipid and the neutral lipid. The results agreed well with those given by direct determination on the acetone-precipitated sample.

Cholesterol esters were prepared by chromatography on silicic acid (33). In this method, the esters are washed through the column without being strongly adsorbed. The only precautions taken to prevent oxidation were to flush the column with nitrogen before starting and to use nitrogen pressure to speed up the flow rate. As pure cholesterol esters dissolve only with difficulty in the reagent used for alkali isomerization, they were always saponified and the isolated fatty acids used for all determinations.

Triglycerides were obtained from a preparation of lipoproteins of density 0.96 to 1.00 by removal of phosphatides from the gross lipid by acetone precipitation and Florisil adsorption and chromatographing the residual neutral lipid on silicic acid. The cholesterol esters were first washed through as previously described. The triglycerides were then eluted with chloroform and the phosphatide reprecipitated at 4°C.

RESULTS

In any study of unsaturated lipids, care must be taken to prevent oxidation. Once the lipid has been extracted from a lipoprotein, it may be stored under nitrogen in the cold for long periods without noticeable oxidation occurring. However, during preparation of the lipoproteins it is not practicable to exclude air at any stage. Since preparation of the lipoprotein fractions used in this study took about 7 days, an attempt was made to prevent oxidations by removing divalent ions from the samples and maintaining the latter at low temperatures. A sample of β-lipoproteins was kept at 4°C under air for 36 days. The spectrum in sodium chloride solution in the range 320 to 500 μm was read at 0, 15, and 36 days. The results (Fig. 1) show that in the presence of ethylenediaminetetraacetate the preparation was remarkably stable. Gurd (34) had found that if ethylenediaminetetraacetic acid were not added, the carotenoid peaks between 440 nm and 500 nm gradually disappeared and the absorption at wave lengths below 300 nm greatly increased (almost 3-fold in 30 days).

Lipid Composition—The composition of the lipid from each lipoprotein group is given in Table I.

These figures agree with others reported for similar preparations (3, 4, 35, 36). The mean ratios of total sterol to phos-
phosphatide are: α-lipoproteins, 0.55; β-lipoproteins (ρ = 1.03), 1.12; β-lipoproteins (ρ = 0.96 to 1.00), 0.58. These fall within the limits given by Oncley (37) for preparations of α- and β-lipoproteins made by several workers.

The predominance of triglyceride in the very low density β-lipoprotein is reflected in its high fatty acid content. The higher density β-lipoprotein (ρ = 1.03) and the α-lipoproteins contain roughly the same percentage of fatty acids. The highest iodine number is given by that fraction which contains the largest proportion of sterol. The lipids of the other two lipoprotein groups have very similar iodine numbers. However, since the lipids of the β-lipoproteins of lower density contains a much higher percentage of fatty acids than that of the α-lipoproteins, the fatty acids of the former must be more saturated.

Several workers have made estimates of the triglyceride content of the α-lipoproteins, but for the most part without direct determinations. According to Oncley (38) about 10% of the lipid could be triglyceride. Other workers have put the figure at between 10 and 20% (3, 4, 35) of the lipid. On one purified preparation (see below) of α-lipoproteins an attempt was made to determine this figure directly. The phosphatide was removed by acetone precipitation and Florisil adsorption as described under “Experimental Procedure.” The neutral lipid was then saponified and after diluting the saponification mixture with water and acidifying with hydrochloric acid, the nonsaponifiable fraction and the fatty acids were extracted into ether. The aqueous phase was assayed for glycerol by the method of Karnovsky and Brumm (39). From this figure it was found that 8% of the lipid was triglyceride calculated as tristearin.

The percentages of peptide in the β-lipoprotein preparations of higher and lower density were 19.1 ± 0.82 and 6.1 ± 0.67, respectively. These figures are somewhat lower than those given by Oncley et al. (5). As these workers did not remove chyomicrons from their preparation of lower density one would expect their peptide results to be lower than those given here. The discrepancy may be due to the different methods used in the determinations or to the loss of some of the protein during the extraction procedures used in these studies. The figure obtained for the peptide present in the α-lipoproteins was 79.5 ± 2.69. This is much higher than the accepted figure (49 to 59%). The difference could be due to inefficient lipid extraction or to contamination by another protein. Examination by paper electrophoresis showed that the preparation obtained by the method previously described was heavily contaminated by albumin. It is known that it is very difficult to separate these two components of plasma, and Bradon et al. (35) state that a much longer centrifugation time is needed than was used here. When part of an α-lipoprotein preparation was purified by repeated ultracentrifugation and the pure preparation examined and compared with the original, the percentages of peptide were found to be 52.3 and 72.2, respectively.

The fatty acid composition of the three lipoprotein groups is given in Table II.

The most striking fact about Table II is the over-all similarity of the three fractions. The percentages of dienes, pentacenes, hexacenes, and hexacenes are lower in the lipoproteins of density 0.96 to 1.00 than in the two more dense lipoprotein groups. This may be due to the very high content of triglycerides. Because of the low and somewhat variable tricenic acid level, no significant conclusions can be drawn from its distribution in the three groups. The percentages of tetraenoic, pentaenoic, and hexaenoic acids are highest in the lipoprotein containing the most phosphatide, i.e. the α-lipoproteins.

These figures harmonize well with the figures given by Hammond and Lundberg (10) and Evans et al. (9) for fatty acids of whole plasma, the percentage values for pentacenic and hexaenoic acids falling between the values given by these two groups of workers. The ratio of pentacenic to hexaenoic acid is lower, however, than that found by them.

Gillies et al. (14) analyzed the S, 0 to 20 and S, 20 to 400 lipoprotein classes from one plasma sample. The results obtained differed markedly from those given above. The figures for the fraction of S, 0 to 20 should correspond to those for our β-lipoproteins of density 1.03, and those for S, 20 to 400 to those for our β-lipoproteins of density 0.96 to 1.00. Gillies et al. (14) found that polysaturated acids were virtually absent from their S, 20 to 400 fraction. Although we find that the percentages are much lower in the β-lipoproteins of lower density than in those of higher density, all of the polysaturated acids are present. These workers also found more saturated acids and less mono-, di-, and trienoic acids in their S, 0 to 20 preparation than we do in our preparation of β-lipoproteins, density 1.03.

The fatty acid contents of the various lipid subfractions are given in Table III. Each analysis represents one original plasma sample.

The results agree well with the general conclusions reached from Table II. Thus the phosphatides from the α-lipoproteins and the more dense β-lipoprotein contain, in general, more tetra-, penta-, and hexacene acids than do other fractions. The fatty acid contents of the phosphatides of the two lipoprotein groups are very similar although it does seem that the phosphatide from the β-lipoproteins of greater density contains more saturated plus monounsaturated acids and less polysaturated acids than that from the α-lipoproteins.

The results agree well with those of James et al. (13) for phosphatides of whole plasma except for the monoenic acid figure which is higher. These workers used gas-liquid chromatography to separate the fatty acids. A much higher proportion of dienoic acids in plasma phosphatides was reported by Lipsky et al. (12) who used chromatography on silicic acid for the separation. However, they analyzed only one plasma sample.

The figures for sterol esters agree well with those of Tuna

Table II

<table>
<thead>
<tr>
<th>Fatty acid composition of lipids of lipoproteins*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipoprotein preparation</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>α-Lipoproteins</td>
</tr>
<tr>
<td>β-Lipoproteins (ρ = 1.03)</td>
</tr>
<tr>
<td>β-Lipoproteins (ρ = 0.96 to 1.00)</td>
</tr>
</tbody>
</table>

* Results are expressed as percentages of the total fatty acids present in the lipid of each lipoprotein. The mean of five observations is given in each case. Variation is expressed as standard error of the mean.
et al. (11) for cholesterol esters of whole plasma. There is a very high content of dienoic acid in these esters and mono- and di-unsaturated acids account for about 70% of the total acids of the sterol esters. Since only one sample of sterol esters from an α-lipoprotein preparation was analyzed, too much significance should not be attached to the very high proportion of trienoic acid and the higher proportion of monoenoic than dienoic acid in this fraction.

More than 80% of the fatty acids of triglycerides from the β-lipoproteins of density 0.96 to 1.00 are saturated or mono-unsaturated. This agrees with previous results on triglycerides (12) of plasma. The one noticeable feature is the very low percentage of tetraenoic acids. A comparison of the results of James et al. (13) with those of Tuna et al. (11) shows that the percentage of this acid is lower in plasma total neutral lipid than in plasma cholesterol esters. This would support our finding that triglycerides contain only a small proportion of tetraenoic acids.

James et al. (15) recently published the results of an analysis by gas-liquid chromatography of the fatty acids of lipoprotein lipids. They did not purify their lipoprotein Fractions in the ultracentrifuge and so their preparations do not correspond to ours. Comparison of our results with theirs is also rendered difficult by the small number of samples analyzed in each case and the consequent lack of information on the variation between individuals. It would seem that the methods we used may have overestimated the monoenoic acids and underestimated the saturated acids.

As a check upon the validity of the methods used in calculating the amounts of saturated and mono-unsaturated acids, two lipid samples were analyzed by gas-liquid chromatography. The samples chosen were (a) triglycerides from a preparation of β-lipoproteins of lower density; (b) total lipid from a preparation of samples chosen were (a) triglycerides from a preparation of samples were analyzed by gas-liquid chromatography. The monoenic acids were overestimated and the dienoic acids underestimated. This would support our finding that triglycerides contain only a small proportion of tetraenoic acids.

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### Table III

**Fatty acid composition of various lipid fractions of lipoproteins**

<table>
<thead>
<tr>
<th>Lipoprotein preparation</th>
<th>Lipid fraction</th>
<th>Saturated acids</th>
<th>Monoenoic acids</th>
<th>Diene acids</th>
<th>Tetraenoic acids</th>
<th>Pentaenoic acids</th>
<th>Hexaenoic acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Lipoproteins</td>
<td>Phosphatides (a)</td>
<td>19.12.4</td>
<td>10.12.11.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphatides (c)</td>
<td>33.225.6</td>
<td>24.92.3</td>
<td>9.02.92.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sterol esters (c)</td>
<td>19.136.4</td>
<td>30.67.8</td>
<td>4.90.50.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Lipoproteins (p = 1.05)</td>
<td>Phosphatides (a)</td>
<td>14.31.8</td>
<td>8.92.11.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphatides (b)</td>
<td>29.344.7</td>
<td>14.11.3</td>
<td>7.41.61.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphatides (e)</td>
<td>42.732.1</td>
<td>17.41.2</td>
<td>3.71.01.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sterol esters (b)</td>
<td>39.320.4</td>
<td>44.81.5</td>
<td>6.01.00.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sterol esters (c)</td>
<td>25.628.8</td>
<td>35.42.2</td>
<td>2.6.40.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Lipoproteins (p = 0.96 to 1.00)</td>
<td>Triglycerides (b)</td>
<td>31.550.7</td>
<td>14.01.3</td>
<td>1.40.60.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triglycerides (c)</td>
<td>38.242.1</td>
<td>11.68.1</td>
<td>1.50.40.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Results are expressed as percentages of the total fatty acids in each fraction. The acids in the cholesterol esters were obtained by saponification and the various determinations done directly on them. It was assumed that 71% of the phospholipids and 98% of the triglycerides were fatty acids. (a), (b), and (c) represent three different original plasma samples used.

### Table IV

**A comparison of results obtained by computation from analytic data or by gas-liquid chromatography for fatty acid composition of lipids from lipoproteins**

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Total lipids α-lipoproteins p = 1.05</th>
<th>Triglyceride of β-lipoproteins p = 0.96 to 1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkalii isomerization and computation</td>
<td>Gas-liquid chromatography</td>
</tr>
<tr>
<td>Saturated</td>
<td>41.3</td>
<td>47.8</td>
</tr>
<tr>
<td>Monoenoic</td>
<td>29.5</td>
<td>28.1</td>
</tr>
<tr>
<td>Diene acids</td>
<td>29.2</td>
<td>24.1</td>
</tr>
</tbody>
</table>

* Results expressed as percentage of sum of saturated, monoenoic and dienoic fatty acids.
† See "Experimental Procedure."

They were chromatographed on a Pye gas-liquid chromatograph with the use of a tube 0.4 feet long packed with 20% polyethylene-5,5-methylpentaihexyl adipate on a stationary phase of Celite (100 to 120 mesh).

Quantitative measurements were possible only on palmitic, stearic, oleic, and linoleic acids. From these figures, the relative amounts of saturated, mono-unsaturated, and polyunsaturated acid were calculated and compared with the results obtained by the methods previously described (Table IV).

Although the very close agreement found in the case of the triglycerides is probably coincidental, the experiment shows no major difference between the two methods. Agreement between the results of alkali isomerization and gas-liquid chromatography was also found by Tuna et al. (11) in their studies of plasma lipids and cholesterol esters.

The close agreement found for the monoenoic acid content in both cases is somewhat surprising, since comparison of our results with those of James et al. (15) and of Nelson and Freeman (36) suggested that discrepancies might be revealed in this estimation.

### DISCUSSION

Fatty acids containing up to six double bonds are present in all three lipoprotein groups and in all subfractions of these groups that were examined. The variations in the fatty acid contents of these lipoproteins seem more to reflect the differing proportions of phosphatides, sterol esters, and triglycerides than specific differences between the lipoprotein groups. This is also indicated from the data of Nelson and Freeman (36) who have examined the fatty acid composition of separated phosphatide species from lipoprotein groups. Large variations between individual donors were found. Sufficient information is, however, not available as yet for any generalisations to be made.

It is interesting to note that the sterol esters contain little more of the fatty acids with three to six double bonds than do the triglycerides from the β-lipoproteins of lower density. Deductions made from iodine number determinations may be misleading. Thus the iodine numbers of the fatty acids of phosphatides and cholesterol esters may be very similar whereas the actual compositions differ greatly. In both sterol esters and triglycerides, the proportion of mono-unsaturated acids is higher than that of the saturated acids but in phosphatides, the ratio is variable.

It is difficult to make deductions about transport of fatty
Acids on the basis of concentration in various lipids. Although it has been known for many years that cholesterol esters of plasma contain a very high proportion of linoleic acid, no evidence has been obtained that cholesterol is important as a vehicle for the transport of this acid (41). The very high content of highly unsaturated acids in the phosphatides may mean that they are transported in this form.

No definite role has been assigned to the very unsaturated fatty acids in the animal body although some of them are essential dietary constituents. Recent work has shown that phosphatides containing high proportions of very unsaturated acids are active in blood clotting systems (42–44). This activity is dependent upon the high degree of unsaturation of the fatty acids since oxidation of the double bonds reduces it (45). The activity seems to be due to the physical properties bestowed on the phosphatide molecule by polyunsaturation (45). An important phenomenon involving the lipid moieties of lipoproteins is the dynamic equilibrium which exists between these entities and the lipids of cellular components of blood (e.g. 13, 46, 47). The large proportion of polyunsaturated fatty acids in the lipids of plasma lipoproteins raises the question of whether these substances are particularly involved in exchanges of lipid moieties such as those mentioned.

SUMMARY

An examination of the lipid moieties of α-lipoproteins of density 1.09 to 1.14, β-lipoproteins of density 1.03, and γ-lipoproteins of density 0.96 to 1.00, has been carried out, particularly in respect to their fatty acids. Fatty acids containing up to six double bonds were found in all three lipoprotein groups. Protections of these entities against oxidation during preparation of the lipoproteins was achieved through the use of ethylenediaminetetraacetate. The proportions of triglyceride, phosphatide, and sterol esters in the lipids from the lipoproteins were determined, and in some cases the nature of the fatty acids in these subfractions was studied with respect to their unsaturation. A limited comparison of data obtained by chemical methods and that obtained by gas chromatography was made, and the agreement found to be good.

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REFERENCES

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