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ABSTRACT

The infrared absorption spectra of some ultracentrifugally isolated serum lipoproteins and those of some related lipids have been presented and discussed. In a lipoprotein spectrum the relative intensities of the ester carbonyl band and certain strong bands of proteins can serve as a rough gauge of the lipid/protein ratio.

Other absorption bands have been correlated with those of the principal component molecules making up the lipoprotein.

A rudimentary method for quantitative analysis of these molecules and of lipid mixtures by infrared methods has been described. Needed improvements have been suggested.

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By means of ultracentrifugal techniques developed in this laboratory and reported previously[1] it is now possible to classify the lipoproteins of blood serum on a density scale. In this scheme a particular molecular species is characterized by its rate of flotation in a given medium and under a specified centrifugal force field. These flotation classes undoubtedly include the same lipoproteins that have been distinguished by other methods and called α- and β-lipoproteins. While the precise relationship between these two classifications has not been completely established, it seems evident that both α- and β-lipoproteins consist of more than one ultracentrifugally characterizable species. The manner of designating these species will be described later in this paper.

In studying the chemical constitution of these lipoproteins, some exploratory work has been done with infrared absorption spectrometry. The purposes of this paper are to present some of the significant results, to discuss their potential usefulness, and to indicate the directions that may be taken in further investigations.

Experimental

Centrifugal Isolation

Since these methods have been described elsewhere,[1] only a brief outline will be given here in order to describe the substances being investigated. In routine practice in this laboratory the serum density is adjusted in such a way that on preparative ultracentrifugation for 13 hours at 81,000 g

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the low density (β) lipoproteins accumulate (free of higher density serum proteins) in the top fraction, which has a resultant solvent density of 1.0630 (at 26° C). Individual species subsequently isolated from this group are designated by their S_f* rates in a medium of that density. The lipoproteins of higher density are prepared in solutions containing heavy water and sodium nitrate for which analogous S_f rate scales have not been employed. These high density species are believed to correspond to the α-lipoproteins. They have hydrated densities of 1.075 and 1.125 and have been tentatively designated L and T, since their migrating boundaries are leading and trailing, respectively, in the analytical ultracentrifuge pattern.

Sample Preparation

Since the absorption of infrared radiation by water is extremely intense, it is necessary to study the lipoproteins in the dry (or nearly dry) state. Evaporation of a lipoprotein solution on a silver chloride plate at room temperature is a satisfactory method of preparing a specimen, provided the salt concentration is not too high. No effort has been made to fix an allowable upper limit of salt relative to lipoprotein, but excessive salt causes a rough or crystalline deposit and consequent loss of radiation by scattering. To obtain the spectra of the more dense species of lipoproteins, the high salt concentrations (required for their preparation in the ultracentrifuge) were reduced nearly to zero by dialysis against distilled water. Extended or repeated dialysis tended to cause precipitation.

The common technique of mulling in mineral oil may be employed to obtain the spectra of dried lipoproteins in the presence of sodium chloride; but sulfates, nitrates, etc., introduce undesirable absorption bands of their own. (Bands at about 7.2 and 12μ in Fig. 1B are believed to be due to residual nitrate ion, incompletely removed by dialysis). For certain purposes the oil-mull technique may prove to be useful.

Molecules of about S_f 20 and higher can be driven to the top of a centrifuge tube in such a way that they separate out in paste form. Often by this means they may be concentrated sufficiently with respect to both water and salt that the resulting paste can be examined directly as a film squeezed between two silver chloride plates. In some cases further drying is either necessary or desirable.

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* Svedberg units; subscript indicates flotation.

Spectrometric Measurements

All of the spectra were recorded with a Baird Associates Model B double-beam spectrophotometer equipped with a sodium chloride prism. Thickness of the lipoprotein films prepared as described above are not accurately known, but the amounts of dry substances in the films are approximately 1-2 mg per cm^2. The carbon disulfide solution spectra were all run in a cell of 0.5 mm thickness and at concentrations in the range from 10 to 30 mg/cc. Gaps in the solution spectra are regions obscured by carbon disulfide absorption.

Reference Materials*

1. β_1-lipoprotein: Obtained by the Cohn fractionation procedure, and provided to us by Dr. J. L. Oncley of the Department of Physical Chemistry, Harvard Medical School.
2. Ovalbumin: Recrystallized three times.
3. Dimyristoyl lecithin: Synthetic compound prepared by Dr. Erich Baer of the Banting Institute, University of Toronto.[2]
4. Egg lecithin: Reprecipitated ten times with acetone.
5. Sphingomyelin: Two samples of purified sphingomyelin from different sources gave virtually identical spectra. One was furnished by Prof. H. E. Carter, Division of Biochemistry, University of Illinois; the other by Prof. S. J. Thannhauser of the Boston Dispensary, New England Medical Center.
6. Cholesterol: Commercial, recrystallized.
7. Cholesteryl palmitate: Prepared in this laboratory by direct esterification according to Page and Rudy.[3]
8. Vegetable oil: Commercial refined salad oil.

Results and Discussion

Lipoprotein Spectra

In Fig. 1 are shown the spectra of some representative lipid-bearing aggregates isolated from human serum by preparative ultracentrifugation. The L, T, and S_f 6 lipoproteins are normal serum constituents and appear to be definite molecular species insofar as the moving boundary criterion

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* The authors are greatly indebted to Drs. Oncley, Baer, Carter, and Thannhauser for the materials generously provided by them.

is applicable. Lipoproteins having S_f values greater than 20 are either transient or variable constituents which contribute to lipemia. These vary from time to time and from individual to individual. Often the ultracentrifugal pattern allows the assignment of specific S_f values greater than 10; however the peaks are apt to be broad, and for purposes of isolation it is generally more convenient to consider a class of molecules defined by an S_f range. This practice is illustrated by the designation of a typical sample as S_f 40-100. Chylomicrons have S_f rates of about 40,000 and may not be lipoproteins in the usual sense, but are included as a centrifugally isolable substance which represents a logical extreme of the lipoprotein sequence.

The spectra in Fig. 1 are given in the order of increasing flotation rates of the substances represented. The order is also that of increasing lipid content. There are two prominent absorption bands which serve to indicate this progressive change in molecular composition. One is the absorption by ester carbonyl groups at 5.8μ, this band being comprised of contributions by glycerides, cholesteryl esters, and certain phospholipids, including the most abundant ones--the lecithins. The other is the absorption band of C-H groups at 3.4-3.5μ, mainly attributable to fatty acid chains (of the various esters) and cholesterol. Both of these bands increase in relative intensity in going down the series. The principal protein absorption bands (See Fig. 4) are those of the peptide group: N-H stretching at 3μ; C = O stretching at 6.1μ; and a third band exhibited by N-monosubstituted amides at about 6.5μ, which has been tentatively assigned to an N-H bending vibration.[4] (It should be mentioned at this point that sphingomyelin, which is believed to be a minor constituent of the molecule, absorbs at 6.1 and 6.5μ also because it is an amide rather than an ester. Its spectrum has been included in Fig. 4). The intensities of these bands relative to the C-H and ester C = O bands diminishes in the same sequence. This crude index of the lipid/protein ratio is probably the most significant inference that can be drawn from the spectrum of an "intact" lipoprotein, although other indications regarding the types of lipids may be discernible and will be discussed below.

A comparison is afforded in Fig. 1C between the spectra of similar lipoproteins prepared in different ways. The sample designated "β_1-lipoprotein" is a film dried from material obtained originally in paste form (see under reference materials). It was found by ultracentrifugal analysis

to consist principally of molecules with a flotation rate of about S_f 6 and smaller amounts of species S_f 6-20. The S_f 6 molecule is the most abundant lipoprotein species in normal human serum. The two spectral curves are qualitatively similar in that the same absorption bands are present in both with about the same relative intensities. (The apparent difference in overall absorption results from the difference in film thickness).

Another pair of samples which are ultracentrifugally similar is represented in Fig. 1D. These are moist films, and the presence of water is indicated by strong absorption at 3μ, 6μ, beyond 11μ, and a low transmission level throughout.

In Fig. 1E the spectrum of centrifugally prepared chylomicrons is compared with that of a representative vegetable oil. The latter is a film between sodium chloride plates, whereas the paste of moist chylomicrons was dried to a film on silver chloride. The sloping background which is apparent in the first portion of the chylomicron spectrum is a result of the combined effects of the silver chloride backing plate and the light-scattering properties of the depositied film. The only other significant difference is the occurrence in the chylomicron spectrum of an absorption band at about 10.3μ. This band might be explained if the chylomicronemia had been produced by the ingestion of margarine or other hydrogenated fat. Such fats are partially elaidinized,[5] and the resulting double bond configuration (geometrically trans) is characterized by infrared absorption at 10.35μ.[6] Natural unsaturated fatty acids (geometrically cis) and their esters in general do not exhibit this band.

Molecular Constituents

In order to interpret the absorption bands which are not patently assignable to localized configurations of atoms (i.e., C-H, C = O, etc.), direct comparisons are made with the spectra of the known major lipoprotein constituents. This has been facilitated by a simple fractionation of the lipoprotein with solvents. By such means the protein and lipids may be separated and their individual spectra compared with those of reference materials. The extraction has been performed by allowing the appropriate solvent to flow over the dried film and collecting the washings in a small beaker. The solvent was evaporated off and the extract dissolved in CS_2 for running its infrared absorption curve. Using egg lipoprotein as an example, the results of such an extractive procedure are illustrated in Fig. 2.

There are shown the spectra of a lipoprotein film, the film residues after each successive extraction, and the two lipid fractions removed from the original material. It was anticipated that acetone would remove fat, cholesteryl esters, and cholesterol, and the mixture of alcohol and chloroform would take out phospholipids. In Figs. 3 and 4 the spectra of some lipoprotein fragments are compared with those of appropriate reference materials. The suitability of any of these reference materials is dependent on spectral constancy within the class it represents. We have studied the infrared spectra of several fats and cholesteryl esters and found that (in CS_2 solution) differences in fatty acid composition do not appreciably alter their major characteristics. For example, the cholesteryl esters of lauric, palmitic, stearic, oleic, and linoleic acids all show a pair of absorption bands at 9.7 and 9.9μ which are not exhibited by any glycerides. Likewise dimyristoyl, dipalmitoyl, and distearoyl lecithins all have essentially similar spectra; and as may be seen in Fig. 3, they are also virtually indistinguishable from purified egg lecithin. The spectra of different proteins have an overall similarity also. With the spectral resolution employed here, and for the purpose of distinguishing protein from lipid, they may be considered identical.

The spectrum of the acetone extract from S_f 40-100 lipoprotein resembles that of the glycerides, although by chemical analysis it is known to contain small amounts of cholesterol, both free and esterified. From the spectra of mixtures it has been shown that the presence of a 3-fold excess of fat can effectively mask the 9.9μ absorption band of cholesteryl esters. Hence it can only be stated qualitatively that fat predominates in the extracted mixture. The acetone-soluble material from S_f 6 lipoprotein however gives a curve (Fig. 3) which is essentially that of a cholesteryl ester plus the contribution of a band at 9.5μ from unesterified cholesterol. In this curve small amounts of fat may escape detection.

From all fractions of human serum the acetone-insoluble fractions appear to yield similar infrared spectra. These resemble the spectra of both synthetic and egg lecithins, and this observation is in agreement with the reported phospholipid composition of human serum (ca 80 percent lecithin).[7] The spectra of cephalins[8] and sphingomyelins are such that these compounds would be difficult to identify in a mixture containing an excess of lecithin. All three have similar strong absorptions in the 8 and 9-9.5μ

regions which are undoubtedly attributable to the phosphoric acid ester structure.[9] Cephalins do not have a distinct band at 10.3μ; and, as mentioned above, sphingomyelin has amide bands at 6.1 and 6.5μ rather than the ester band at 5.8μ.

The spectrum of the protein residue serves not only for identification, but to show whether or not the lipids (esters) have been completely removed by the solvents. In one of the two protein residue curves in Fig. 4 a weak ester carbonyl band is still present at 5.8μ, revealing the presence of a small amount of unextracted lipid. Further washing usually removes such residual lipids.

Analytical Applications

It is possible to make a semi-quantitative estimate of lipoprotein composition in terms of its major constituents by the use of intensity measurements at appropriate spectral positions in the absorption curves of Fig. 2. If after evaporation of solvents the lipid extracts are dissolved in measured volumes of CS_2 and the spectra obtained, concentrations of the individual components in these solutions may be determined from suitable calibration curves (Absorbance vs. concentration). From these data the actual quantities of extracted lipids can be calculated. The acetone-soluble lipids are calculated as cholesteryl esters or as fat, whichever is indicated at predominant by a visual inspection of the curve. If a distinct band appears at 9.5μ, unesterified cholesterol may also be estimated. A spectrophotometric three-component analysis of this fraction has been considered, but the overlapping of principal ester absorption bands at 5.8 and 8.6μ and the relative weakness of others makes this an unfavorable system for this treatment. Furthermore small amounts of phospholipid are undoubtedly present in this extract. The acetone-insoluble lipids are calculated as lecithin. In order to determine the amount of protein, a lecithin-protein ratio is obtained from the relative intensities of the 5.8μ and 6.5μ absorption bands in the spectrum of the acetone-extracted residue. This necessitates a calibration of these band intensities from a series of lecithin-protein films of differing composition. If now the sum of the measured components is regarded as the approximate total amount of material originally present in the film, a percentage composition can be obtained in terms of protein, phospholipid, esters, and in some cases unesterified cholesterol. If the content of unesterified cholesterol

is low enough that it cannot be estimated, its neglect will not greatly alter the percentage figures for the other components. Sphingomyelin is also neglected.

While useful results have been obtained by this procedure in the study of isolated lipoproteins, particularly where only small samples are available, it is perhaps equally important as an illustration of the potential usefulness of infrared spectrometry in lipid analysis generally. With a view toward the development of an integrated system of lipid analysis from this approach, we have attempted to analyze lipid extracts from serum, serum fractions, lymph, etc., by essentially the same procedure. In these cases, however, only the solution spectra of the two lipid fractions are needed, since proteins have been excluded from the system. Similar results have been obtained, which can at best be regarded as semi-quantitative. It is pertinent to point out the shortcomings, therefore, and the areas in which improvement is being sought.

1. Further evaluation of materials to be used as reference standards is needed. Spectral variability within classes must be re-examined more critically as accuracy improves in other respects.
2. Consideration should be given to the presence of minor constituents and of contaminating substances which may be unidentified. A prerequisite for a successful quantitative analysis by infrared methods is a knowledge of all constituents in the mixture and the character of their absorption spectra. Lipid extracts from serum have been found to contain substances which so far have not been fully characterized.
3. Probably the most serious defect of the method is the inadequacy of the separation achieved by the crude extraction procedure employed. It has been found that as much as 15 percent of the total phospholipids can be carried into the acetone-soluble fraction; and in cases of films which contain relatively large amounts of protein there is often evidence of "acetone-soluble" lipids in the phospholipid fraction. Current effort is being directed toward the accomplishment of better separations by adsorption techniques such as have been reported recently by Borgstrom.[10] A sufficiently good separation will either avert the problem of multicomponent analysis, or at least yield sets of lipids that are more amenable to this treatment. It should also aid in the elimination of extraneous interfering substances.

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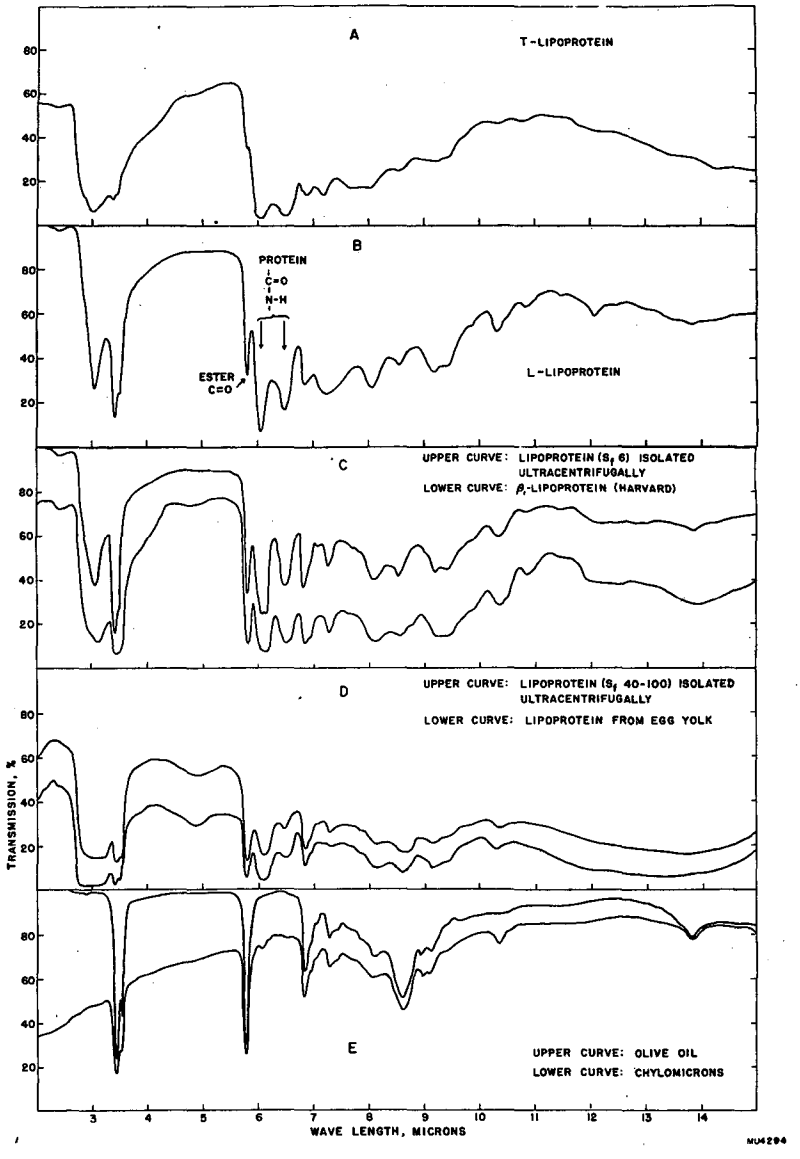

Fig. 1

Infrared spectra of principal lipoprotein species isolated from human serum by preparative ultracentrifugation. Comparisons with similar entities are shown in C, D, and E. Samples prepared as films on silver chloride plates, with the exception of olive oil. The latter is a film between salt plates.

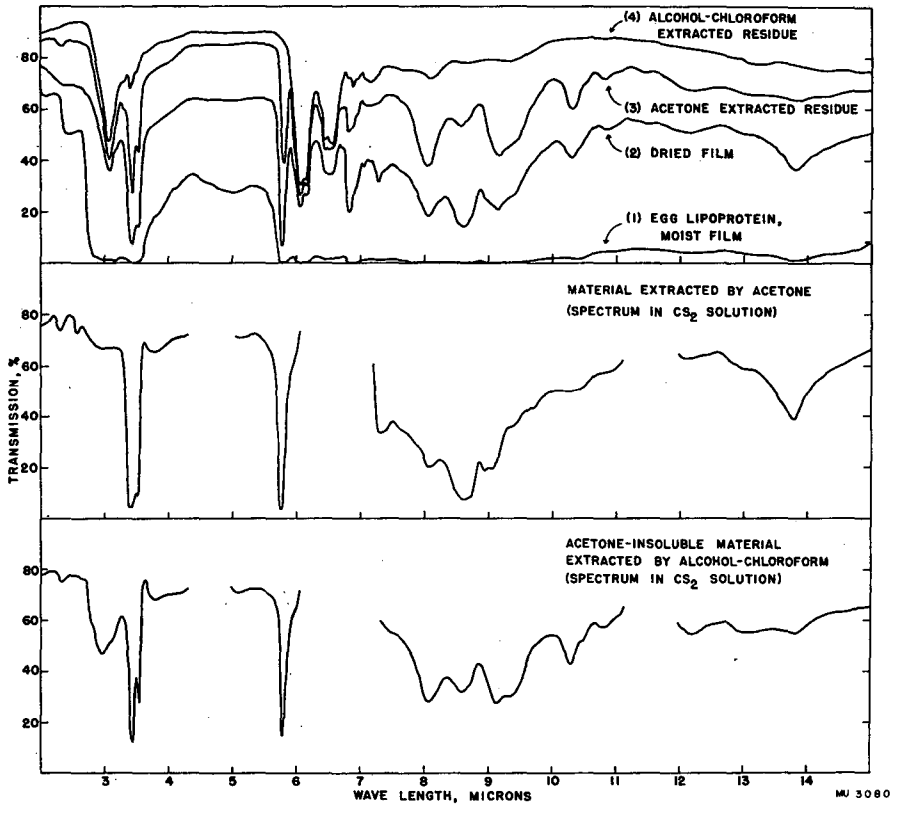

Fig. 2

Infrared absorption curves of substances obtained in serial extractions of a lipoprotein film with acetone and 1:1 alcohol-chloroform.

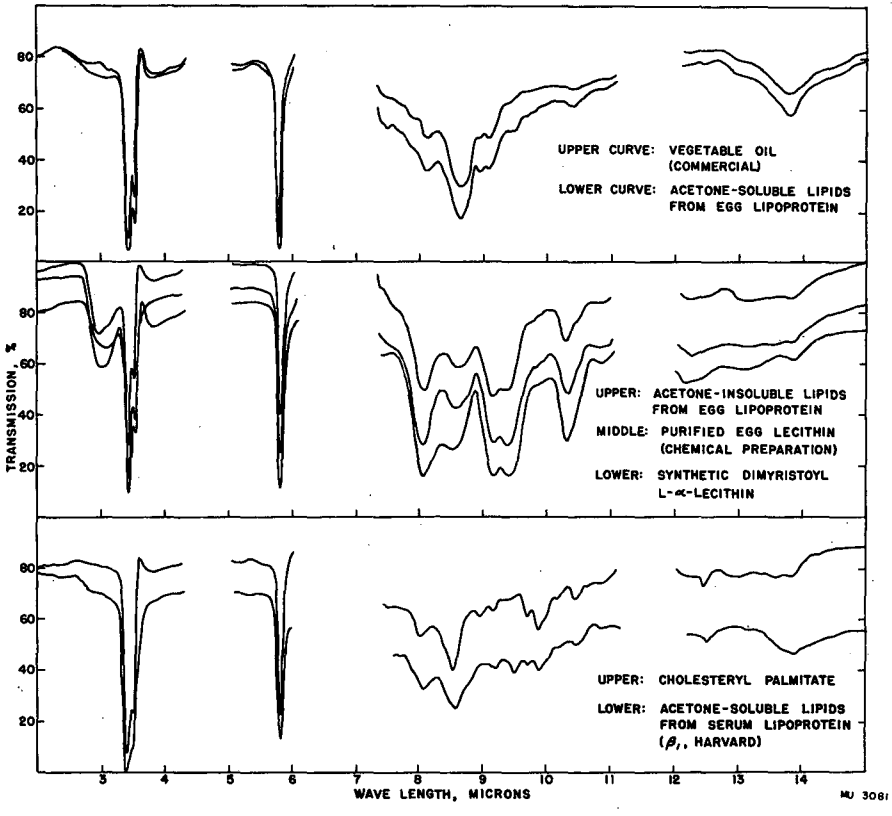

Fig. 3

Comparison of the spectra of extracted lipids with spectra of reference compounds. Spectra in CS_2 solution.

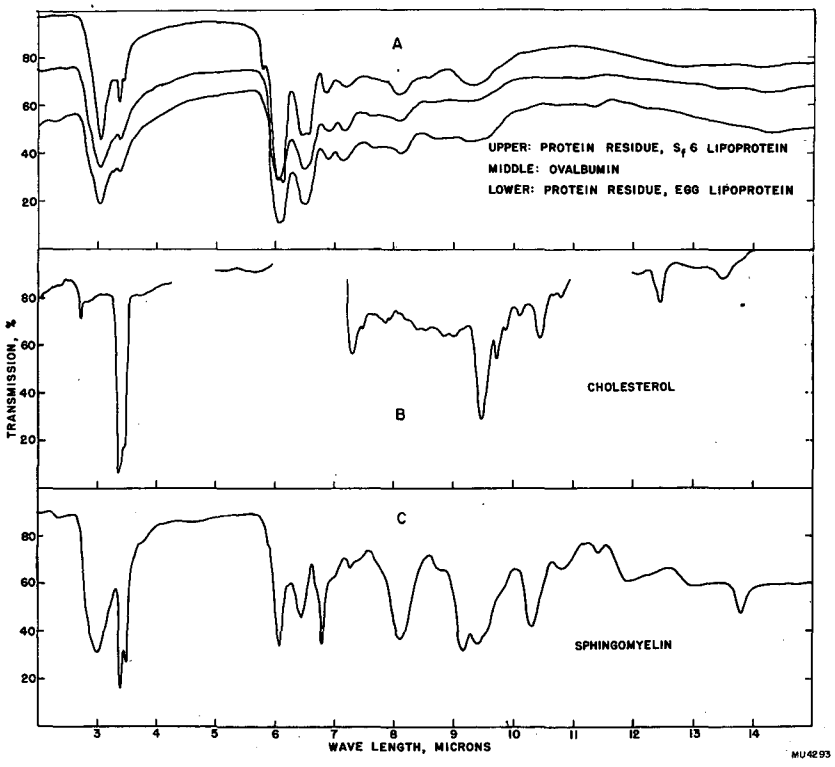

Fig. 4

A: Comparison of the spectra of protein residues from extracted lipoproteins with the spectrum of a simple protein (ovalbumin)

B, C: Spectra of other reference compounds which are present as small fractions of the total lipoprotein molecule. Cholesterol in CS_2 solution; sphingomyelin as a solid film.