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INFRARED SPECTROPHOTOMETRIC METHODS FOR THE ANALYSIS OF BLOOD LIPIDS

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INFRARED SPECTROPHOTOMETRIC METHODS FOR THE ANALYSIS OF BLOOD LIPIDS

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I. INTRODUCTION

Among the various physical and spectroscopic tools that have been adopted by chemists in the last two decades, one of the most widely useful is infrared spectrometry. It has a number of favorable attributes which have led to its intrusion into almost all branches of chemistry, both theoretical and applied. Perhaps the most fundamental of these attributes is the high content of information about molecular structure that is inherent in the infrared spectrum of a substance. The spectrum is a display of the frequencies of radiation that are absorbed, and in the infrared these correspond to frequencies of internal vibration of the molecules. From a theoretical point of view, mathematical analysis of the vibrational spectrum leads to a description of the modes of vibration, and permits the establishment or verification of certain architectural features of individual molecules. In most applied or analytical usage however, the infrared spectrum is used empirically. That is to say it is one of the observable properties of a substance which may be compared with the corresponding established properties of known substances as an identification procedure. (Or the measurement of absorption intensity at some selected spectral position may be made the basis of a quantitative analysis.) In the so-called fingerprint technique one searches a collection of spectra of known compounds to find a matching spectrum for that of the unknown. No knowledge or interpretation of the spectral content may be required in this case, and the matching

process is often made the task of a machine or computer. Although the fingerprint technique is quite general in principle, its successful application depends somewhat on the richness and variability of spectral detail. These qualities do not characterize the spectra of the long-chain fatty acids and their derivatives, since the differences resulting from additional methylene groups or double bonds may not be easily perceptible. Furthermore, in dealing with a limited group of well-known lipid classes, it soon becomes possible to recognize the spectral features of each class without the necessity of searching through a large file of reference curves. For the present purpose then we will have no further concern with either the purely theoretical (mathematical) analysis of spectra or the purely empirical approach based on a reference catalog. General reference works on infrared spectroscopy may be consulted for background in those areas (1,2,3,4,5).

The spectra that are presented and discussed in a later section are those of representative types of blood lipids. They are illustrative of the spectral patterns that are encountered, and in a limited sense they can be regarded as a reference collection. However it is equally important to consider the significant spectral features which are interpretable from group correlations. Group correlations are the established characteristic frequencies of absorption associated with various bond types, functional groups (hydroxyl, carbonyl, ether, etc.) or structural features (*cis*, *trans* isomers, rings, etc.). Knowledge of the characteristic frequencies of such groups enables one to recognize the spectral indications of them, and thus to make valuable inferences about the chemical structure of a substance, even if a complete identification is not possible from the spectrum alone. Condensed summaries of group correlations are available

in the form of tables or charts, the most widely distributed being that of Colthup (6). Copies are obtainable from instrument manufacturers. Such charts should be used with some caution, with due regard for the complexity of the spectrum and for alternative assignments of a given absorption band. A complete and detailed discussion of group frequencies has been given by Bellamy (7,8).

From the point of view of structural investigation, Chapman's treatise on the structure of lipids (9) includes a thorough discussion of infrared spectrometry as well as a number of other physical and spectroscopic tools. This valuable reference work deals extensively with basic physical properties such as modes of vibration, molecular configuration, crystal forms, thermal effects, etc. and their relationships to spectroscopic observations. Other reviews of the infrared spectroscopy of lipids are by O'Connor (10), Schwarz (11,12), and Freeman (13,14). O'Connor's review contains an extensive table of group frequencies that are pertinent to lipid spectra. This table has also been reproduced by Chapman (9).

In addition to the qualitative aspects of lipid spectra, we will be concerned with their application to quantitative analysis. Basically the same spectrophotometric principles hold as for measurements in the visible and ultraviolet regions. However differences in sampling considerations and techniques need to be pointed out. The specific types of blood lipid analyses that have been done and may be done will be reviewed.

The methodology of infrared spectrometry is fairly well established. After about three decades it has reached a plateau of development where further innovations are infrequent. Commercial spectrophotometers and accessories are

available in some variety, and our discussions of such instruments will be limited. Factors bearing on applications will be indicated, with very little reference to optical principles or mechanical design details. Modes of sample handling need more emphasis, however. These techniques are quite general, and most of them are potentially useful in lipid work. Some — such as gas sampling, for example — may be used very little if at all; others will be considered according to their importance in lipid work.

Much of the information derived for lipids in general is relevant to the blood lipids in particular. The latter are in fact limited to a fairly small number of compound types. Cholesterol, cholesteryl esters, glyceryl esters, and three principal kinds of phospholipids ordinarily comprise more than 95% of the lipids in blood. These major lipids, together with the minor lipid constituents in blood and a few significant related compounds, will be the focus of attention in this chapter. The intent is to provide a concise package of information based on infrared spectrometry that is of practical interest to the lipid chemist whose work involves these classes of compounds.

II. METHODS OF OBTAINING SPECTRA

A. Instruments

The infrared spectrum of a substance is represented as the graph of its transmittance (of radiation) *vs.* wavelength over some defined interval. Although the word infrared might imply any wavelength beyond the red end of the visible spectrum, the range of most common interest extends from about 2.5 μm[1] to about 16 μm. In frequency (reciprocal wavelength) units[2] these limits correspond to 4000 cm^{-1} and 625 cm^{-1}. This range includes most of the fundamental

modes of molecular vibration of organic molecules. It is the most thoroughly studied and documented; and except where otherwise specified it is the range denoted by the term "infrared spectrum" throughout this article.

The spectral region lying between the visible limit and 2.5 μm is usually called "near infrared". Absorption bands observed in this region are much weaker, since for the most part they are overtones (harmonics) or combinations of fundamental frequencies. Experimentally, near infrared spectra are obtained by apparatus and techniques more nearly akin to visible range spectrometry. Although they are generally less useful than infrared spectra of the fundamental region, some potential applications in the lipid field have been discussed by Holman *et al*. (15,16).

There are instruments which are capable of measuring spectra beyond 16 μm and some distance into the far infrared, but there is at present very little of such data that is pertinent to lipids and no further mention will be made of it.

For the spectral range that has been designated as infrared, there is available a variety of commercial spectrophotometers. Although they can be categorized according to their complexity, versatility, performance, or price, they have some common basic elements which will be described briefly. These elements are:

(1) a source of radiation;

(2) a monochromator;

(3) a radiation detector;

(4) a measuring system (which almost always implies recording of the spectrum during a continuous scan).

Another common feature is the use of reflecting optics — front-surface mirrors —

throughout (except for sample cells, detector window, certain filters, and of course a prism if one is used). Radiation sources are thermal, usually a nichrome wire or non-metallic rod heated by an electric current. The energy distribution has the approximate shape of a black-body curve, with its maximum somewhere near the short wavelength (2 μm) end of the infrared range. As the wavelength is increased the energy falls continuously, and it is down by a factor of about 100 at the long wavelength end. To compensate for this decrease, the spectrometer slits must be correspondingly widened so that the available energy remains adequate for measurement.

In the monochromator the focused radiation from the source enters through a slit, is collimated and then dispersed in space by a prism or a diffraction grating. The spectrum thus formed is refocused in the plane of an exit slit, which allows the passage of only a narrow wavelength interval. The location of the emerging spectral band is determined by the geometry of the system and can be varied over the whole range by changing the angular relationship of the prism or grating in the monochromator. The width of the interval, which is basically related to the resolution to be achieved, is determined by the dispersing power of the prism or grating and by the actual slit width.

The output radiation from the monochromator may be of the order of microwatts or less, and a very sensitive thermal detector is required to measure it. In most commercial spectrophotometers a radiation thermocouple is used. Some less common detectors are bolometers, pneumatic detectors and semiconductor devices, the latter being largely limited to special purpose instruments. In the detector the incident energy is converted to an electrical signal, which is amplified and used to drive the recording system.

In order to facilitate continuous and automatic recording of spectra, there are some additional features embodied in most infrared spectrophotometers. A double beam system is usually employed, in which the source radiation is directed along two paths. The sample is placed in one path and an appropriate reference or blank in the other. The two beams are pulsed alternately into the monochromator at a suitable frequency, and the intensity in each beam generates its own signal. If they are equal the transmittance is recorded as 100% (ratio of sample beam energy to reference beam energy is unity). If there is excess absorption in the sample beam the difference in intensities gives rise to an alternating signal in the detector which (when amplified) causes a servo-system to adjust an attenuator in the reference beam to restore intensity balance. Motion of the attenuator drives the recorder pen to a position on the scale corresponding to the absorption in the sample beam. If all other losses in the sample beam (such as cell absorption, solvent absorption, surface reflections, scattering etc.) are suitably balanced out the value indicated by the pen is sample transmittance. In recording a spectrum over the specified range, then, the wavelength is changed continuously by rotation of the grating (or Littrow mirror in a prism instrument). At the same time the slits are being opened to maintain a constant order of background energy; and the recorder chart is being synchronously driven so that the abscissa corresponds to the wavelength (or frequency) being passed by the monochromator. The transmission is measured continuously during the scan and recorded as the ordinate value on the chart. It should be pointed out that the scanning speed may affect the resulting curve, and in general it should be slow enough so that the recording system has time to respond.

The rudimentary outline just given for an infrared spectrophotometer applies to both simple and complex instruments. In the simplest types the operating parameters (scanning speed, slit width schedule, amplifier controls, etc.) are preset for some suitable average set of conditions for obtaining spectra, and this may be adequate in ordinary circumstances. In such an instrument operational control is reduced to two or three switches. More versatile (and expensive) instruments, on the other hand, provide for considerable variability in all operating factors so that performance can be optimized for any given situation. Energy, resolution, scan speed, and noise level are all subject to some degree of control. In addition, such instruments are expected to be better engineered and to be capable of more accuracy and precision in both wavelength and intensity measurements.

It is an interesting coincidence that the most important part of the fundamental vibration region of the spectrum is nicely encompassed by the effective working range of a sodium chloride prism. The earliest spectrometers employed prisms cut from natural crystals, and this spectral interval became known as the "rocksalt" range. Development of the technology for making high-quality synthetic crystals large enough for prisms was one of the factors which made commercial infrared spectrometers feasible. For a long time prism instruments were prevalent, but in recent years the trend has been toward the use of diffraction gratings - once again as a result of technological advance. The most obvious superior characteristic of gratings is higher resolving power. This is especially apparent at shorter wavelengths where sodium chloride resolution is relatively poor. The adoption of gratings has been to some extent accompanied by a tendency to record spectra on a scale that is linear in frequency

(cm^{-1}) rather than wavelength (μm). In comparison with the linear wavelength scale, the linear frequency scale expands the high frequency region and compresses the low frequency region. It is thus more appropriate for the resolving power of the grating.

The instrumental requirements for applications in lipid chemistry are probably intermediate on the scales of both complexity and cost. Simple instruments may be adequate for simple qualitative identification work, but some degree of flexibility is desirable to meet unusual sampling situations, and for quantitative analysis. The resolving power of the grating can be of significant advanrage for certain purposes, which will be discussed later.

B. Sampling

The general methods of sample handling are applicable to lipids, except that gas phase techniques may be safely ignored. Solutions may be used for either solids or liquids, but the number of useful solvents is extremely limited. The spectra of carbon tetrachloride, carbon disulfide, and chloroform are shown in Fig. 1. Reagent grade solvents are adequate for most purposes, except that the small amount of ethanol normally present in $CHCl_3$ as a preservative should be removed by passage through a silica gel or alumina column. It is evident that in each of the solvents there is at least one region of the spectrum that is obscured by strong solvent absorption. Nevertheless, by taking part of the spectrum in CCl_4 and part in CS_2 the entire range can be covered except for a short interval between 6 μm and 7 μm where both solvents absorb. Bromoform has been suggested as a solvent with which to bridge that gap (17). Solvents whose molecules are more complex or more polar have correspondingly complex spectra and stronger absorption bands and are of little utility. (Water, although a simple molecule, is highly polar and absorbs intensely in the infrared. There are however, some limited regions where it is sufficiently transparent in very thin layers so that partial spectra can be

obtained of substances in water solution. This point is not especially significant in connection with lipids, but may be pertinent to lipoproteins). In spite of these limitations, solutions are generally preferable for quantitative analysis, and absorption bands to be measured can usually be found elsewhere than in regions of strong solvent absorption. Most lipids, except some of the more polar ones, are sufficiently soluble in the acceptable solvents. For qualitative purposes it may often be desirable to avoid the use of solvents, and the techniques for liquid and solid samples will be outlined. These procedures are less convenient for quantitative work, but can be so used for substances which are not sufficiently soluble.

Two types of absorption cells are used for liquids. The first type — called demountable — is assembled and disassembled for each sample. The technique is nevertheless extremely simple. A few drops of liquid are placed on a polished salt (NaCl) plate; a similar plate is set on top to form a "sandwich"; and the assembly is clamped lightly in a suitable frame to be placed in the spectrophotometer. In this instance the thickness of the liquid film is unknown, and controlled only by the viscosity of the liquid and the tightness of clamping. It is sometimes called simply a capillary film. If a thicker film is needed, a spacer in the form of a thin metal washer may be put between the plates, with the sample in the open center. (For a mobile or volatile liquid, a spacer also acts as a seal. It is usually cut from lead foil, but insoluble plastic is sometimes used.) The spacer determines the thickness of the liquid layer, but not precisely enough for good quantitative work. Demountable cells are also available in which the sample thickness is approximately determined by the depth of a shallow depression in one or both windows. Depending on the absorption

properties of the substance, the required thickness is usually in range 0.01-0.05 mm.

The second type of liquid cell — also made with salt windows — is permanently assembled and has a fixed thickness. This type is strongly preferred for quantitative analysis of either solutions or undiluted liquids. In the latter case the path length is usually in the range indicated above, whereas for solutions the cells are usually thicker, ranging up to 1.0 mm or more. These cells have stoppered filling ports, and may be filled from a syringe or a dropper.

Some lipids have the consistency of a soft wax or grease, and these may be squeezed into a thin film between salt plates. Others may be too firm for this procedure but will form a suitable film by evaporation of a solution to dryness on a salt plate. Still others tend to crystallize on evaporation and form layers that are particulate and transmit poorly because of scattering. These may be dealt with by one or the other of two general methods for handling solids.

In the KBr pellet method, a solid sample is finely ground and uniformly dispersed in dry KBr. The mixture is then pressed in a suitable die in a hydraulic press to form a pellet. Grinding and mixing can be done with a mortar and pestle, or in a mechanical device such as a dental amalgamator. The die is usually evacuated, and the pressure required is 80,000 - 100,000 lbs/sq in. Equipment is available from manufacturers of instruments and accessories. Pellets are commonly disk-shaped, but dies are also available for making them rectangular. Blank disks can be made optically transparent, and many types of

samples can be incorporated without significantly reducing their quality. Numerous factors, however, can cause the prepared disks to exhibit some degree of fogginess. If moderate, it may not impair the usefulness of the disk, and the observed effect on the spectrum is a sloping background, with transmission falling as the short wavelength limit is approached. (Some of the KBr pellet spectra of lipids in Section III B illustrate this effect. An attenuator has been used in the reference beam instead of a blank.) This background phenomenon is a result of scattering loss, which can be more severe as the disk is whiter or chalkier. In such a case the transmission may be too low to obtain a spectrum at all. Some possible causes of poor disks are:

(1) particle size too large;

(2) non-uniform distribution of material;

(3) water in sample;

(4) chemical or physical alteration of the sample;

(5) insufficient force on die;

(6) impure KBr;

(7) vacuum not adequate.

Prepared KBr — pure, dry, and of suitable particle size — is obtainable from commercial suppliers. It should be protected from exposure to atmospheric moisture, and some pains may be taken to dry it more exhaustively. However it is very difficult to avoid traces of water in the final pellet, as evidenced by the appearance of two slight dips in the spectrum at about 3 μm and 6.1 μm. (See Fig. 9). Other than these a KBr blank should be free of absorption bands. The pellet method has been discussed critically and in detail by Fridmann (18).

For some purposes freeze-drying is an attractive alternative procedure for preparing a KBr-sample mixture. Since it evidently yields a molecular dispersion that is essentially amorphous, it avoids such difficulties as particle size effects

and crystal polymorphism. The spectral characteristics of a freeze-dried compound are somewhat like those of the liquid state, whereas the spectra of crystals (as prepared by grinding) tend to have sharper, more numerous, and more discrete bands. This difference should be borne in mind (as well as the possibility of polymorphism) in connection with the qualitative spectral identification of solid samples. Their solid-state spectra should be compared only with those of reference compounds prepared in the same way. The freeze-drying method can be recommended for quantitative analysis by virtue of its reproducibility, and also because it is a feasible technique for handling micro amounts of material. Such procedures have been described in detail by Schwarz (12) for tissue lipids, and by Mason (19) who recommend two stages of lyophilization for substances (i.e., lipids) which are not water soluble.

An older method of treating solids is to grind the sample to a fine powder in a mortar and disperse it as a paste or mull by further grinding in a few drops of white mineral oil. This mixture is then squeezed into a film between salt plates. Operationally this procedure is extremely simple, and for many qualitative purposes it is quite adequate. Oil obscures the regions of absorption by CH_2 and CH_3 groups, (hydrocarbon chains of lipids) but substantial and significant portions of its spectrum are relatively transparent (see Fig. 2). As in the case of undiluted liquids, quantitative analysis can only be done with the use of an internal standard.

The amount of sample required when standard instrumentation and cells are used is ordinarily in the range 1 - 10 milligrams. Special cells and accessories are available for working with quantities down to a few micrograms. The first requirement in micro sampling is to reduce the cross-sectional area occupied by

the sample, keeping the thickness and concentration the same. By bringing the sample as near as possible to the slit (or some other focal point), its area may be made comparable to the slit dimensions with little or no loss of energy. Samples well below a milligram can be handled in this way with suitable microcells or small rectangular pellets. Below about 100 μgm the reduced sample area becomes substantially smaller than the minimum beam area and a large fraction of the energy is lost or masked out. In this range it is therefore necessary to employ an auxiliary optical system, usually called a beam condenser. Such a device may be constructed with either transmitting or reflecting optics; and it provides in the sampling space an image of the source which is reduced in dimensions by a factor of 1/3 to 1/6. The beam energy is thereby concentrated in this smaller area, and still smaller cells or pellets can be used. Various auxiliary systems of this kind are provided by manufacturers of instruments and accessories. Pellets as small as 0.5 mm in diameter can be made. A micro technique of this sort is needed for the spectroscopic examination of recovered fractions from either gas-liquid or thin-layer chromatography.

If gas chromatography fractions are to be examined by infrared spectrophotometry, it is usually for the purpose of identification, since quantitation will have been already done. Depending on the amount and volatility of the material in the fraction, it may be collected in a simple solvent trap or cold trap as it emerges from the chromatograph, and subsequently transferred to a suitable absorption cell. Direct collection of liquids in a microcell of one microliter capacity is made possible by a special accessory (Barnes Engineering Co., Stamford, Conn.). A simple trap for direct collection in a KBr powder bed has been described (20) and commercial devices are available (Barnes Engineering

Co., _supra_, and Carle Instruments, Inc., Anaheim, Calif.). Both of the latter are designed so that the KBr is already in a die, ready for pressing a pellet immediately after the fraction is collected.

In thin layer chromatography, fractions are recovered by scraping or aspirating the powder from the desired spot, extracting the lipid material with a suitable solvent and incorporating it into a pellet in one of the usual procedures. (It has been noted by Rouser _et al._ (21) that some solvent systems can solubilize material from thin-layer adsorbents and contaminate lipid eluates. Such extraneous material can be removed by filtration through a small Sephadex column.) Various techniques have been described in the literature (22,23). A novel device for introducing small samples into KBr is called the WICK-STICK (Harshaw Chemical Co., Cleveland, Ohio). This is a small triangular prepressed bed of KBr which is placed upright in a small vessel together with a solution of the sample. On evaporation of the solvent the sample concentrates in the upper tip of the triangle, which is then broken off and made into a pellet.

III. QUALITATIVE APPLICATIONS

A. Interpretation of Infrared Spectra

Depending on the particular problem at hand, the lipid chemist may wish to use the infrared spectrum to identify an isolated pure compound, either specifically or as to type; or he may wish to characterize mixtures with respect to probable constituents; or he may wish to show the presence or absence of some particular chemical group or structural feature. Specific identification brings into play the catalog of reference spectra for direct comparison. Otherwise the background information consists basically of the group correlations, which have been mentioned earlier. Before describing the lipid spectra

in these terms, it is perhaps worthwhile to discuss some general features of infrared spectra which guide the approach to their interpretation. The following brief outline of these features is tailored to the understanding of lipid spectra. For example, the spectral characteristics of aromatic compounds are not included. For a more extensive and detailed discussion of characteristic group absorptions see Refs. 7,8.

For the purpose of "reading" an infrared spectrogram — in the sense of assigning structural groups to observed absorption bands — it is helpful at the outset to realize that the infrared range can be subdivided according to a broad classification of vibrational types. A tabulation of these subintervals is approximately as follows:

2.5 - 4.2 μm (4000 - 2400 cm^{-1}): hydrogen stretching vibrations (OH, NH, CH, SH bonds)

4.2 - 6.5 μm (2400 - 1550 cm^{-1}): multiple bond stretching vibrations (C ≡ C, C ≡ N, C = C, C = N, C = O)

6 - 16 μm (1667 - 625 cm^{-1}): hydrogen bending vibrations and single (skeletal) bond/stretching vibrations (C - C, C - O, etc.)

The occurrence of the hydrogen stretching vibrations at the high frequency end of the spectrum is a logical consequence of considering a molecule as a system of coupled harmonic oscillators. As derived from Hooke's Law, the frequency of a single oscillator is given as

$$\nu = \frac{1}{2\pi}\sqrt{\frac{f}{M}}$$

where f is the restoring force per unit displacement and M is the reduced mass

of the system. If the oscillator is a diatomic molecule, f is the force constant of the bond between the atoms, and M is expressed as the product of the individual atomic masses divided by their sum. When one of the two atoms is hydrogen, the value of M is much smaller than in a molecule composed of two heavier atoms; so for comparable force constants the vibration frequency is higher. Substantially all of the significant hydrogen stretching frequencies fall within the range indicated; and nearly all other vibrations are excluded, with the possible exception of weak overtones or combinations of lower frequencies.

All C-H bonds do not have the same single characteristic stretching frequency, represented in the absorption spectrum by a single peak. Rather there is in the spectrum of a (non-aromatic) hydrocarbon a pattern of absorption bands that is determined principally by methylene (CH_2), methyl (CH_3), and olefinic hydrogen (C = C-H) groups. In both CH_2 and CH_3 groups the stretching vibrations can be symmetric or antisymmetric. Thus each group has two frequencies, and four bands would be expected in the spectrum of a simple linear alkane. These are only partially resolvable however, and in NaCl prism spectra two peaks are observed at about 3.4 μm and 3.5 μm (2920 cm^{-1} and 2860 cm^{-1}). The first is a composite of the antisymmetrical vibrations of both groups, and the second is the corresponding mixture of symmetrical vibrations. In a branched alkane there is at least one tertiary hydrogen (R_3CH), whose absorption is relatively weak and also unresolvable. Stretching of a hydrogen on a double-bonded carbon atom has a slightly higher frequency, which is variable with double bond type: 3.25-3.35 μm (3100-3000 cm^{-1}). The peak is observable but still poorly resolved in the NaCl prism spectra of olefins.

Grating spectra show fairly good resolution of this peak. Some other distinguishable types of C-H bonds are acetylenic: C ≡ C-H (3.05 μm, 3300 cm^{-1}) and aldehydic: O = C-H (3.65 μm, 2730 cm^{-1}).

The hydroxyl (O-H) stretching frequency is strongly influenced by hydrogen bonding. For a very dilute solution in a nonpolar solvent, where little or no hydrogen bonding takes place, the absorption peak is sharp, at about 2.75 μm (3640 cm^{-1}). As the concentration (and hydrogen bonding) is increased the peak broadens and intensifies, and its position moves to longer wavelength. In the spectra of solid or undiluted liquid alcohols the band is quite broad, and its center is in the vicinity of 3.0 μm (3300 - 3400 cm^{-1}). It is in fact similar to the O-H stretching band of liquid water. An extreme case of hydrogen bonding is found in carboxyl groups, which associate pairwise to form cyclic dimers. In these structures the hydroxyl of each mating molecule is hydrogen bonded to the carbonyl group of the other. The strength of these hydrogen bonds is such as to shift and broaden the absorption peak even more than in alcohols. The O-H stretching band of carboxyl extends from about 3 to 4 μm (3300 - 2500 cm^{-1}) and is seen in the spectra of fatty acids as broad shoulders on both sides of the C-H stretching bands.

N-H stretching frequencies are very close to the O-H frequencies, and they are similarly affected by hydrogen bonding, although not as strongly. Unassociated N-H groups absorb in the range 2.85 - 3.05 μm (3500 - 3300 cm^{-1}). When involved in hydrogen bonding, they absorb between 2.95 and 3.2 μm (3400 - 3100 cm^{-1}). In the NH_2 groups of primary amines and amides, there are also symmetric and antisymmetric stretching modes (just as in CH_2), giving rise to two distinct frequencies in these ranges. The presence of a positive charge

on the nitrogen atom, as in amine salts or amino acids, usually has a further complicating effect on the spectrum, depending on the type of compound. Quite commonly new bands are observed at longer wavelengths, between 3.6 and 5 μm (2800 - 2000 cm^{-1}); but the possible effects are too varied to discuss in detail here.

The types of multiple bonds which are of some significance in relation to blood lipids are C = C and C = O. Triple bonds probably do not occur in mammalian lipids, and their absorptions are well segregated in the spectrum below 5 μm. The C = N double bond is also unlikely to be encountered, and can for the most part be disregarded. Its stretching frequency falls in approximately the same narrow range as that of the C = C double bond — 5.95 to 6.15 μm (1680 - 1625 cm^{-1}).

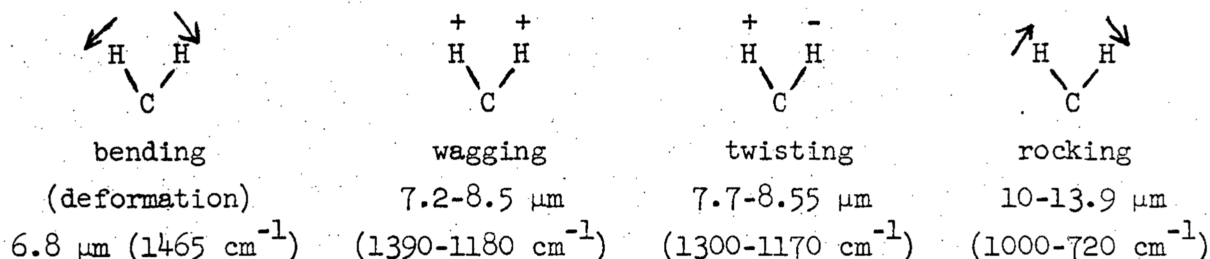
The C = C stretching absorption is in fact relatively weak. In methyl oleate (Fig.5) for example it appears to be about two orders of magnitude weaker than the C = O band at 5.75 μm (1740 cm^{-1}). This comparison illustrates the dependence of absorption intensity on the magnitude of the dipole moment fluctuation which is associated with the vibrational motion of the atoms. The C = O bond is strongly polarized, whereas the C = C bond has a symmetrical charge distribution. If the C = C bond has <u>cis</u> configuration a small dipole can be generated by stretching. If it is <u>trans</u>, the electrical symmetry is maintained during the vibration and there is no absorption band at all (see methyl elaidate, Fig. 4). If the C = C double bond is part of a conjugated system its frequency is lowered and its intensity of absorption is increased.

The carbonyl absorption band is an important feature of infrared spectra generally and of lipid spectra as a class. Because it is relatively free from

interference by other absorptions, it has been thoroughly studied, and its frequency ranges established for the various classes of carbonyl-containing compounds. Such other spectroscopic aspects as absolute intensities, solvent effects, differences between liquid and solid state, etc., have also been investigated. All C = O absorptions occur within the limited range 5.4 - 6.5 μm (1850 - 1540 cm^{-1}). Between 5.4 and 5.95 μm virtually no other groups absorb, so this is exclusively a carbonyl region. It includes most simple (or unconjugated) fatty acids, esters, ketones, aldehydes, anhydrides, and acid chlorides. Between 5.95 and 6.5 μm may be found the absorption bands of amides, some conjugated carbonyls, and the carboxyl ion. The most common non-carbonyl absorbers in this range are C = C (weak), conjugated C = C (including aromatic), primary amines (NH_2 bending), and organic nitrate.

Patterns of absorption in the third spectral region (6 - 16 μm, 1667 - 625 cm^{-1}) can be quite complex, and for many classes of molecules only a few of the bands observed there can be assigned to group vibrations. Those for which correlations are best established include hydrogen bending and certain single bond stretching modes. Hydrogen bending implies deformation of bond angles by motions of the hydrogen atoms perpendicular to the bond directions. An example, the NH_2 group, has a bending vibration frequency at about 1610 cm^{-1} (6.2 μm); and its occurrence there is the principal reason that the bending range overlaps the double bond region. The C-H bending modes, which are of primary importance in lipids, are distributed over a wide range. As in the case of the stretching vibrations, these are best described as motions of CH_2, CH_3, and C = C-H groups.

If a CH_2 group were to exist as a detached molecule, it would have only one bending mode — the angle deformation. As part of a chain of CH_2 groups linked by carbon-carbon bonds, its hydrogen atoms can execute four distinct types of in-phase motions which can be designated as bending modes. These are sketched as follows:



bending (deformation) 6.8 μm (1465 cm^{-1}) — wagging 7.2-8.5 μm (1390-1180 cm^{-1}) — twisting 7.7-8.55 μm (1300-1170 cm^{-1}) — rocking 10-13.9 μm (1000-720 cm^{-1})

A half-cycle of each vibration is shown by the directions of motion of the H atoms, + indicating upward from the plane of the page and - indicating downward. The absorption band corresponding to the deformation mode appears at about 6.8 μm (1465 cm^{-1}) and is a prominent feature in the spectra of all long-chain compounds. The frequency of this vibration is lowered in a methylene group that is adjacent to a polar group or a double bond. If the polar group is a carbonyl (ester or fatty acid), the band is at 7.1 μm (1410 cm^{-1}). This is also the position of a weak carboxyl absorption. For a CH_2 group adjacent to a double bond the absorption is at 6.95 μm (1435 cm^{-1}) and tends to be overlapped by the main CH_2 band.

In theory, if a hydrocarbon chain is in the fully extended, planar zig-zag form, the frequencies of the other bending vibrations occur as sequences distributed over a considerable spectral range. This is a result of interaction between the methylene groups, and involves the phase relationships among the individual methylene motions along the chain. The spectra of

long-chain compounds in the crystalline state show such band sequences, whereas those of liquids do not. (It is presumed that in the liquid state, the chains are not constrained to the planar zig-zag conformation, and the collective interaction among methylene groups is not definable.) A prominent characteristic of the spectra of solid fatty acids and their esters is the "band progression" between about 7.2 and 8.5 μm (1390-1180 cm^{-1}). These band sequences in saturated straight-chain acids were analyzed by Jones _et al_. (24) and by Meiklejohn _et al_. (25), who minimized band interference by using fatty acid soaps. As _n_ (the number of methylene groups) increases, the spacing between bands decreases, and there are approximately _n_/2 bands in the interval. The bands have been assigned as the methylene wagging modes, and in liquid or solution spectra they degenerate into a single weak band in the vicinity of 7.7 μm (1300 cm^{-1}). In lipids this band is often overlapped by stronger absorptions and is not readily discernible. The twisting modes are believed to occur in the same general frequency range as the wagging modes. They are evidently weaker; their identification and assignment have not been completely reliable; and they have little practical significance.

The rocking modes form a series whose long wavelength limit is at 13.9 μm (720 cm^{-1}). In some instances other bands of the series can be identified, but usually it is only the 13.9 μm peak that is observed. For very short chains (< 4 methylenes) the band is shifted to shorter wavelengths: 13.5 μm (740 cm^{-1}) for propyl side chains and 12.9 μm (775 cm^{-1}) for ethyl side chains. Another very significant observation in connection with the CH_2 rocking frequency is its splitting into a doublet in certain crystalline forms. This has been attributed to interaction between methylene groups of adjacent chains which is

permitted only in a particular orientation.

A methyl group can have a bending vibration that is symmetrical, in which all three hydrogens move inward and outward (with respect to the axis) in phase; or antisymmetrical, in which one hydrogen moves inward while the other two move apart. The latter mode has a frequency very close to that of the methylene deformation, and usually appears as an unresolved shoulder (6.85 μm, 1460 cm^{-1}) on the long wavelength side of the 6.8 μm (1465 cm^{-1}) peak. The symmetrical vibration of CH_3 has a characteristic band at 7.25 μm (1375 cm^{-1}), nicely separated from the other alkane absorptions. Whenever two methyl groups are attached to the same carbon atom, their interaction results in a splitting of this band into a doublet. This is a useful diagnostic feature in relation to chain branching.

The bending vibrations of hydrogen on a double-bonded carbon atom can be classified as planar or nonplanar with respect to the C = CHR plane. Absorptions attributable to planar motions occur in the rather narrow range 7.05-7.15 μm (1420-1400 cm^{-1}). The band is only strong when the structure is $R_2C = CH_2$, which occurs infrequently in the types of lipids with which we are concerned. For RHC = CHR (cis) the absorption is weak or moderate; and for RHC = CHR (trans) it is zero (forbidden by symmetry). Since in fatty acids and their derivatives the α-methylene group absorbs at the same wavelength these bands are not likely to be very useful.

The out-of-plane bending vibrations of olefinic hydrogen, on the other hand, are quite distinctive for each configuration. For unconjugated double

bonds the structural correlation is as follows:

$RHC = CH_2$	10.1 μm, 11.0 μm (990 cm^{-1}, 910 cm^{-1})
$R_2C = CH_2$	11. 25 μm (890 cm^{-1})
RHC = CHR (trans)	10.35 μm (965 cm^{-1})
RHC = CHR (cis)	~ 14.25 μm (~ 700 cm^{-1})
$R_2C = CHR$	11.9-12.5 μm (840-800 cm^{-1})

The first three types listed give strong, sharp, specific bands. The cis double bond absorption is broader, less well defined, and it overlaps the CH_2 rocking absorption at 720 cm^{-1}. The band for trialkylethylene is of only moderate intensity, also broad and more variable in position. Although these correlations do not apply to conjugated double bond systems, further distinctions of those structures can be made (26).

The principal single bond stretching vibrations in lipids are C-C and C-O. The C-C bonds are mostly links in long alkyl chains and are subject to coupling interactions. This leads to a series of bands, just as for the coupled methylene group vibrations of long chains. Theoretically the C-C stretching sequence falls in the range between about 8.7 μm and 11.5 μm (1150-870 cm^{-1}). The number of vibrational modes is equal to the number of C-C bonds, but only some of these are active (allowed by symmetry) in the infrared. Because the dipole moments generated by these motions are very small, absorption is very weak and the bands are difficult to identify — especially in the longer chains. Since the C-C bands can also be easily obscured or confused with other absorptions in the spectra of non-hydrocarbons, they are of no practical utility in the interpretation of lipid spectra.

The ester group is a common feature of lipids; and its C-O stretching vibrations are responsible for prominent absorption bands in the range 8-10 μm (1250-1000 cm^{-1}). Usually a strong band is observed between 8 μm and 8.7 μm (1250-1150 cm^{-1}) and a weaker one between 8.7 μm and 10 μm (1150-1000 cm^{-1}). In the spectra of acetates the strong band is characteristically near 8.05 μm (1245 cm^{-1}), and its position is relatively independent of the remainder of the molecule. This absorption is believed to be associated with the C-O bond nearest the carbonyl (although the two C-O bonds are coupled through the oxygen atom and the vibrations must interact). For large acyl groups (non-aromatic) this band moves to longer wavelengths and in lipid spectra it is in the vicinity of 8.6 μm (1165 cm^{-1}). The weaker band appears in the spectra of acetates between 9.45 and 10 μm (1060-1000 cm^{-1}). It is poorly characterized, however, and its identification is uncertain in many ester spectra.

The C-O stretching vibration of alcohols has a wavelength range from about 8.5 μm to 10 μm (1175-1000 cm^{-1}). In simple noncyclic saturated alcohols a frequency correlation exists for primary, secondary, and tertiary structures. Their absorptions occur at about 9.5 μm (1050 cm^{-1}), 9.0 μm (1110 cm^{-1}) and 8.75 μm (1140 cm^{-1}) respectively. Allylic alcohols have a band at about 9.9 μm (1010 cm^{-1}). Cholesterol is a cyclic secondary alcohol and does not conform to that correlation, but has a C-O absorption at the primary alcohol position.

In simple saturated ethers the C-O-C group absorbs strongly in the vicinity of 9.0 μm (1110 cm^{-1}). This characteristic band position is shifted to about 8.35 μm (1200 cm^{-1}) in vinyl ethers, so that it may be difficult to distinguish if ester groups are also present.

The various phospholipids are phosphate diesters. Strong absorptions of this structure are the P=O stretching band at about 8.1 - 8.3 μm (1235-1215 cm^{-1}) and bands attributable to P-O-C stretching between 9 μm and 9.5 μm (1100-1050 cm^{-1}). The P=O frequency falls outside the range designated for double bonds because of the larger mass of the P atom relative to carbon. The P-O-C bands are close to the C-O absorptions of alcohols and ethers, and if a band of either of the latter groups is present it may be overlapped or obscured by the phosphate spectrum.

B. Spectra of Lipid Types

Figs. 3 to 12 are the spectra of lipid compounds which are of interest in the study of blood lipids. They have been chosen to be representative of the various lipid classes, and to illustrate their spectral characteristics. Differences between solid-state and liquid or solution spectra are also exemplified. Most of the compounds were obtained from commercial suppliers, as designated. The phospholipids were stated to be 96-98% pure, other lipids at least 99%. Purities were not verified, but wherever possible the spectra were checked against known or previously published curves. Film thicknesses quoted are rough estimates on the basis of band intensities.

The spectra were recorded on a Perkin-Elmer Model 421 double-beam, filter-grating spectrophotometer. A scale change mode was employed, in which the normal linear frequency scale is contracted by a factor of two between 4000 cm^{-1} and 2000 cm^{-1}, and expanded by a factor of two between 2000 cm^{-1} and 625 cm^{-1}. The resulting curves resemble more closely the spectra recorded on a linear wavelength scale and are visually more nearly comparable with prism spectra that appear in most of the earlier literature. Expansion of the low frequency range can also be rationalized on the basis that this region contains the absorption patterns of principal qualitative significance. Relatively low resolution conditions (slit program = 1000 x 2) were used, but the observed

resolution in the C-H stretching region (3.3 - 3.6 μm) is significantly better than NaCl prism resolution. (This would be more clearly shown if the scale were not compressed in this region.)

Normally the recorded curve exhibits transient spikes at frequencies where the filter or grating is automatically changed (2500 cm^{-1}, 2000 cm^{-1}, 1580 cm^{-1}, 990 cm^{-1}). These spikes have been deleted, in some cases by raising the pen off the paper momentarily during recording, otherwise at the stage of photographing the original curves. This is noted only to explain an occasional irregularity in the curve at one of these frequencies. Such irregularities are of little consequence except at 990 cm^{-1}, where the recovery time from the transient is especially slow and the curve itself is apt to be changing more rapidly. In some spectra there may be a slight distortion of the curve at that point.

The curves were recorded directly on small charts (9 cm x 18.75 cm) for convenience in reproduction. There is some loss in accuracy in this procedure, but the curves are believed to be correct within 5 cm^{-1} on the frequency scale between 2000 cm^{-1} and 625 cm^{-1}, and 20 cm^{-1} between 4000 cm^{-1} and 2000 cm^{-1}. These limits are comparable to reading errors on the reduced scales, and are probably adequate for most qualitative comparisons. Most of the wavelengths (frequencies) quoted in the text are approximate. Allowances must be made for the broadness of some bands, for variations in the calibration of different instruments, and for fluctuation among values taken from the literature. There may also be small differences between liquid and solid states, and in solvents of different polarity.

In the solution spectra, regions of total absorption by the solvent have been omitted. Where there are other strong solvent bands (e.g., ~8 μm and ~10 μm in CCl_4) the reduced energy may cause slight blurring of the spectra.

C. Useful Features of Lipid Spectra.

The most significant group correlations that are relevant to lipid spectra have been discussed in a general way in part A of this section. It may be profitable now to pinpoint some of the specific spectral characteristics that are most useful in the qualitative analysis of lipids. Aside from the identification of a lipid by simple recognition of its overall spectral pattern, various structural questions may be put regarding double bonds, chain length, branching, ester type, etc. The spectrum provides answers of varying degrees of completeness, depending in part on whether the sample is an isolated pure compound, a mixture of homologues, or a mixture of classes. The basic information is to be found in the appropriate group correlations. Moreover these correlations can be better defined for the limited set of lipid compounds if the reference spectra of pure lipids are examined against them as a background. There are some features in the spectra which distinguish various classes of lipids from one another. Other features persist through all or most of the classes because they are associated with the hydrocarbon chain moieties.

The fatty acids have some unusual absorption bands that are attributable to their dimerized structure. One of these is the absorption of the strongly hydrogen bonded hydroxyl of the COOH group. This is a broad band extending from about 3 to 4 μm (3300 - 2500 cm^{-1}); and appears in the spectrum (e.g., Fig. 3) as broad shoulders on both sides of the sharper C-H stretching bands. The hydroxyl hydrogens — because they are constrained in the ring structure formed by the dimer — can also oscillate in a direction perpendicular to the plane of the ring. This non-planar bending mode is responsible for a characteristic fatty acid absorption at about 10.7 μm (935 cm^{-1}). The C = O

stretching frequency for the dimerized form in solution is observed at about 5.85 μm (1710 cm^{-1}). At high dilution, however, a band of the monomer may also appear at a higher frequency (ca 1760 cm^{-1}). The spectrum of a soap has not been shown, but ionization of the carboxyl group shifts the carbonyl absorption into the range 6.2 - 6.45 μm (1610-1550 cm^{-1}). This band is quite strong.

Fatty acids in unesterified form are normally present in blood as a very small percentage of the total lipid. Consequently they are not detected spectroscopically unless they are isolated — or an enriched fraction is obtained — by some separation procedure. If the free fatty acid level in serum is elevated pathologically, or by lipid degradation during long periods of storage, it may become manifested in the total lipid spectrum as a shoulder on the side of the ester band.

With few exceptions, viz., fatty acids, cholesterol, and sphingolipids, the major lipid constituents in blood are esters. If is therefore important to know how the characteristic absorption bands of the ester group vary from one type to another. The peak frequencies of the C = O band and the major C-O-C band in solution spectra of the significant lipid classes are listed in Table I . Data for fatty acids and methyl esters are also included. Although methyl esters are not normal constituents in blood, they are a convenient derivative form, almost universally used for gas chromatography. Since they are also useful for other analytical purposes, some representative methyl ester spectra have been shown in the previous section (Figs. 4,5). It is noteworthy that the carbonyl frequencies are measurably different for the various ester types in blood. In the spectrum of a total lipid extract from

wavelength region of the spectrum. The five methyl groups of cholesterol enhance the 7.25 μm (1380 cm^{-1}) absorption in comparison with straight-chain compounds. The intensity of this band can be judged in relation to the 6.85 μm (1465 cm^{-1}) band, which on a mass basis is about the same for the two types of compound (cf. triolein, Fig. 7 and cholesteryl palmitate, Fig. 8).

It may be noted that there is considerable detail in the "fingerprint" region (beyond 7.5 μ) of the cholesterol spectrum, and it is virtually the same for solid and solution. This is in contrast to the straight-chain compounds, whose spectra as solids contain numerous sharp bands that are absent from their solution spectra. This difference can be explained on the basis of the rigidity imparted to the cholesterol molecule by its condensed-ring structure. Thus its configuration does not change appreciably on going into solution and its relatively complex spectral pattern is retained. On the other hand, straight-chain compounds, which exist in the crystal as extended planar zigzag chains, undergo deformations in solution (or liquid state) as a result of internal rotations, with a consequent loss of spectral detail.

The infrared spectra of numerous phospholipids appear in the work of Rouser et al. (29) in connection with brain lipid studies. Nelson has presented spectra of phospholipids isolated from plasma and red cells of various species (30,31), and pointed out some of the characteristic spectral features (32). The phospholipids as a class have in common the phosphate ester group, which is characterized in their spectra by a strong P = O absorption band at 8.1 - 8.3 μm (1235-1215 cm^{-1}) and a P-O-C band of about equivalent intensity between 9 and 9.5 μm (1100-1050 cm^{-1}). These two absorptions are the dominant features which distinguish phospholipids from the other lipids. Cerebrosides

absorb strongly between 9 and 10 μm (1100-1000 cm^{-1}) because of their C-O(H) groups, but they can be distinguished from phospholipids by the absence of a P = O band in their spectra.

Within the phospholipid class, those which contain the glyceryl ester moiety show carbonyl absorption at about 5.76 μm (1737 cm^{-1}). The spectra of sphingolipids, which do not have carboxylic ester groups, are characterized instead by the distinctive bands of N-monosubstituted amides at about 6.1 μm (1640 cm^{-1}) and 6.45 μm (1550 cm^{-1})(Fig. 11). The presence of choline in the molecule is empirically associated with a sharp band of moderate intensity at 10.35 μm(965 cm^{-1}). The bond origin of this band is not known, but it may be a C-N vibration which has gained intensity from the charge on the nitrogen atom. There is no absorption band at this point in the spectra of phosphatidyl ethanolamine (Fig. 11), cardiolipin (Fig. 12), or phosphatidyl serine (Fig. 12), and it has also been shown to be absent from the spectrum of N, N-dimethylphosphatidyl ethanolamine (32). In phospholipid mixtures the 965 cm^{-1} band provides a positive means of detecting the presence of choline-containing species, but there is no corresponding band for the ethanolamine group. Indeed, except for indications of choline and the amide group, the spectra of phospholipids are so similar that minor components cannot be detected in mixtures. The curve of lysolecithin resembles that of lecithin except that the ester carbonyl band is somewhat weaker relative to the phosphate absorptions. The ratio should be reduced by half, but this is not obvious in the film spectra shown. The same considerations apply to the spectra of plasmalogen and phosphatidyl ethanolamine (Fig. 11).

About 70% of the phospholipids in human plasma are phosphatidyl choline and about 20% are sphingomyelin, the remaining 10% being composed mostly of

phosphatidyl ethanolamine and lysolecithin. Both major components contain choline and this is reflected in the spectrum of the total phospholipid mixture by the presence of a prominent 965 cm^{-1} band. The relative proportions of lecithin and sphingomyelin can be judged from the intensities of the ester and amide carbonyl bands, bearing in mind that their absorptivities are not equal. As indicated above, the minor constituents cannot be identified without prior isolation. Although phosphatidyl ethanolamine is a more prominent constituent in erythrocyte phospholipids (31) and the plasma phospholipids of some species (30), this is only revealed indirectly by a relatively less intense 965 cm^{-1} band.

In the spectrum of phosphatidyl serine (Fig.12) there is a band of significant intensity at about 6.05 μm (1655 cm^{-1}) whose origin is not entirely clear. Kimura and Nagai (33) studied the spectra of this compound in various forms and observed that the band is absent in the metal-free form. When they prepared monosodium and disodium salts however, bands reappeared at 1645 cm^{-1} and 1610 cm^{-1} respectively. The latter band is quite broad, and more nearly at the expected COO^- position. Thus while the absorption appears to be related to the ionized carboxyl group, the frequency is shifted out of the normal range, possibly as a consequence of some complex interaction among ionized groups.

The presence of absorption bands at about 3 μm (3300 cm^{-1}) and 6.1 μm (1640 cm^{-1}) in the spectrum of lecithin is best accounted for by bound water. These bands appear in the spectra of both natural and synthetic lecithins, but their intensities may vary with treatment.

The separation and identification of individual fatty acids by gas chromatography has largely preempted this area of lipid characterization. Nevertheless it is pertinent to indicate some of the spectral manifestations of fatty acid chain structure. Most of these persist through the spectra of the various lipid classes and are thus independent of lipid type. For example the spectra shown of palmitic acid and its various esters in the solid state all exhibit the same characteristic band progressions between 7.2 μm and 8.5 μm (1390-1180 cm^{-1}). In an isolated species the chain length can be determined from these bands (25).

Some absorptions related to double bond structures have been discussed in Section III A. In blood lipids the double bonds are almost exclusively of the <u>cis</u> unconjugated type. The bands attributable to <u>cis</u> unsaturation may be seen most easily in the spectra of methyl esters (Figs. 4,5). Increasing in intensity with the number of double bonds, they are: C=C-H stretch, 3.3 μm (3020 cm^{-1}); C=C stretch, 6.05 μm (1640 cm^{-1}); C=C-H planar bending, 7.1 μm (1410 cm^{-1}); C=C-H nonplanar bending, 14.2 μm (700 cm^{-1}). In addition, there is a change in the pattern of the methylene bending bands. The decrease in the number of methylene groups is reflected by a weaker 6.8 μm (1465 cm^{-1}) band, while the 6.95 μm (1435 cm^{-1}) band becomes stronger because more of the methylene groups are adjacent to double bonds. These effects are not very pronounced at low levels of unsaturation, but at higher levels they should be detectable in spectra of any of the lipid classes.

Of the other double bond configurations only the <u>trans</u> isomers are of significance in relation to blood lipids. They are present chiefly as a result

of ingestion of hydrogenated fats (or in ruminants by bacterial action in the rumen). Detection is by means of the 10.35 μm (965 cm^{-1}) band in all classes except phospholipids, which have an interfering band. For this class, detection is made possible by conversion to methyl esters. Methanolysis is also helpful in fatty acids and cholesteryl esters when the level of <u>trans</u> isomers is very low.

Branching in fatty acid chains is of secondary interest in blood lipids, although some gas chromatographic investigations have suggested the presence of trace amounts. Spectral indications of branching have been described by Freeman (34), and more extensive and detailed studies of branched-chain acids were made by Fischmeister (35,36). Branching increases the proportion of methyl groups, which results in an increase in the intensity of the methyl band at 7.25 μm (1375 cm^{-1}). If the branching is such that there are two methyl groups on the same carbon atom this band is split into a doublet. There are characteristic bands for ethyl groups (12.9 μm, 775 cm^{-1}) and propyl groups (13.5 μm, 740 cm^{-1}) at the distal end of the chain. Branching near the carboxyl group results in alteration of the pattern of the bands normally appearing at 7.8 μm (1285 cm^{-1}) and 8.1 μm (1235 cm^{-1}).

D. <u>Lipoproteins</u>

There are two aspects to the study of lipoproteins by infrared spectroscopy. One has to do with the intact lipoproteins and the qualitative information that can be derived from their spectra. The other relates to the determination of their lipid compositions by the use of infrared analytical methods.

The spectra of serum lipoproteins as dried films on AgCl plates have been reported (37). Examples of those of high-density (HDL) and low-density (LDL)

classes are shown in Fig. 13. A spectrum of egg lipoprotein (Fig. 14 Ab, from ref. 37) is virtually identical with that of the very low-density (VLDL) class from serum. (These three major classes are defined by their behavior in the ultracentrifuge under specified conditions. See Chapter .) The principal protein bands are those of the peptide (H-N-C=O) group. The N-H stretching absorption appears at about 3 μm (3300 cm^{-1}), the carbonyl stretching at about 6.05 μm (1650 cm^{-1}); and the 6.45 μm (1550 cm^{-1}) band arises from a complex vibrational mode of which N-H bending is a major component. The remaining bands are identifiable with lipid absorptions, and their chemical structure assignments are as shown in the spectrum of serum lipid extract (Fig. 18).

The 1650 cm^{-1} and 1550 cm^{-1} protein bands have been commonly designated as Amide I and Amide II bands, respectively. Their use in the interpretation of protein structure goes back to the work of Ambrose and Elliott (38), who observed different band positions for "folded-chain" (α) and "extended-chain" (β) structures in the spectra of protein films. (The α-helix model had not yet been advanced.) Theoretical considerations of the manner in which the amide group vibrations are affected by chain structure have been discussed by Miyazawa and Blout (39). More recently the frequency correlations have been examined for proteins in D_2O solution by Susi _et al_. (40,41). They have given the following peak positions for the Amide I band in various structures: random coil, 1640 cm^{-1}; α-helix, 1650 cm^{-1}; β (pleated sheet), 1625 cm^{-1}. In addition the antiparallel form of the β-structure has a weaker band at about 1680 cm^{-1} by which its presence can sometimes be recognized.

The spectrum of low-density serum lipoprotein in D_2O solution has been studied by Gotto _et al_. (42). They observed a complex band with its principal

maximum at 1625 - 1630 cm^{-1}, indicating a substantial proportion of β-structure. A distinct shoulder at 1640 cm^{-1} suggested the presence of some random coil, and another at 1680 - 1685 cm^{-1} implied that the β-structure is at least partly in the antiparallel form. A less well-defined shoulder was seen at 1650 cm^{-1}, indicating the possibility of some α-helix content. On addition of sodium decyl sulfate the β-peaks both disappeared, and the principal maximum shifted to 1650 cm^{-1}, the position characteristic of an α-helix. A shoulder at 1640 cm^{-1} (random coil) remained.

The dried film spectra (Fig. 13) do not necessarily reflect properties of the native state of lipoproteins. In the LDL spectrum there appear to be two distinct peaks at about 1660 cm^{-1} and 1625 cm^{-1}. The latter corresponds to the principal absoprtion maximum in D_2O solution, attributed to β-structure. (Amide I frequencies are slightly higher in the solid state than in solution.) In the HDL spectrum however, only one absorption maximum is observed in the Amide I region at about 1650 - 1660 cm^{-1}. Thinner films do not reveal any other peaks, and 1650 cm^{-1} was reported for this frequency in a KBr pellet preparation by Scanu and Granda (43). This band is broad, and may be taken to indicate either α-helix or random coil structure, or a mixture of the two. Scanu and Granda also concluded from circular dichroism measurements that high-density lipoprotein contains a higher percentage (~70%) of α-helix than does the low-density class (~40%).

The infrared spectra of erythrocyte membranes in the form of dried films have been investigated by Maddy and Malcolm (44) and by Chapman <u>et al</u> (45). Except for relatively less intense lipid bands these spectra bear considerable resemblance to the spectra of HDL from serum. Differences in lipid composition,

and also the presence of a carbohydrate moiety, are responsible for small differences in the pattern beyond 8 μm (< 1250 cm^{-1}). The Amide I band appears at 1660 cm^{-1} (44) or 1652 cm^{-1} (45), and no indications of β-structure are seen. Removal of the lipid by treatment with n-butanol does not alter the Amide I band, but heating with 50% ethanol at 70°C for three minutes results in the appearance of a second peak at about 1630 cm^{-1}. The spectrum in the amide region then resembles that of LDL (Fig. 13). The doublet peak, indicating partial transformation to β-structure, was also observed by Chapman *et al.* in the spectra of erythrocyte membranes after treatment with various solvents. However it did not appear as a consequence of temperature change alone (-110° to + 110°C).

In addition to the inferences regarding protein conformation in erythrocyte membranes, Chapman *et al.* (45) studied the 720 cm^{-1} CH_2 rocking absorption in the spectra of dried films. The fatty acid chains in the membranes are those of phospholipids, and the intensity of the CH_2 rocking band is taken as an indicator of the degree to which the (methylene) chains are extended in the all-*trans* zig-zag configuration. In the membrane spectrum at room temperature the band is very weak, indicating very little of this structure. At lower temperatures the extended form is thermodynamically favored, and at -110°C the intensity of the 720 cm^{-1} band is significantly increased. In the spectra of lipid extracts from membranes the same relative intensity changes with temperature are seen; but at any given temperature the band is more prominent in the lipid spectrum than in the membrane spectrum. This observation seemed to reflect a greater degree of disorder in the lipids when incorporated into the membrane structure. Some speculative interpretations of this difference were offered

in terms of possible lipid-protein interactions.

With respect to the chemical composition of lipoproteins, the spectra of dried films provide little more than a qualitative indication of the lipid-protein ratio. This can be judged from the relative intensities of principal lipid bands (C-H, C-O) and protein bands (Amide I and Amide II). These differences are apparent in the various lipoprotein spectra shown in Figs. 13 and 14. Although some tentative inferences about the major lipids are possible from the pattern beyond 8 μm, such spectra are not satisfactory for quantitative analysis. An early attempt to obtain quantitative information from them is exemplified by Fig. 14. In this instance lipids were successively eluted from the lipoprotein film by different solvents, taking advantage of the insolubility of phospholipids in acetone. Spectra of the lipid fractions in CS_2 solution are also shown. The spectrum of the acetone eluate of egg lipoprotein (or VLDL from serum) identifies it as principally triglyceride, whereas from LDL or HDL the corresponding eluates have the spectral characteristics of cholesteryl esters. These eluates are nevertheless mixtures which are not resolved satisfactorily with this method. Furthermore, solvent fractionation under these conditions is probably not reliable, so that this approach to quantitation is not very promising.

Lipoprotein lipids have been analyzed more successfully by extracting them from the isolated lipoprotein fractions and applying quantitative infrared methods as described in a later section. Results obtained by various combinations of these methods have been reported (46,47). The most recent analyses (unpublished) give slightly revised compositions from those previously found, and are believed to be more reliable for the major components. Triglycerides

and cholesteryl esters were determined by the two-component method (48) described in Section IV B. The adsorbed phospholipids from that procedure were recovered and measured spectrophotometrically at the 9.15 μm (1090 cm^{-1}) peak (CCl_4 solution). A direct measurement of unesterified cholesterol is not obtained by this method, but this component is estimated either by difference or from cholesteryl esters, assuming that there is a constant ratio between free and esterified cholesterol. The latter assumption — although acceptable for total serum lipid — is not strictly true for the separate lipoprotein classes. Nevertheless the cholesterol results are more consistent by this method than by difference. When calculated cholesterol values are added to the directly measured components, the average closures with total lipid weight range from 96% to 102% in all classes except VLDL (females). In this class the amount of material available was small (average 1.8 mg of lipid); and therefore the analytical accuracy was less than normal. Closures were more erratic, averaging about 90%. Although unesterified fatty acids may also be present in lipoproteins in very small amounts, they were not measured.

Composition data, determined for the three major lipoprotein classes from 16 normal males and 16 normal females (both nonfasting), are summarized in Table II. The standard deviations indicate that the VLDL class exhibits greater variability in all components. This is consistent with the greater variability in density distribution within this class, but may be due in part to lower analytical accuracy for the small amounts of lipid available. These data are cited here mainly as an illustration of infrared analysis and will not be discussed further. They may be compared with more extensive data on lipoprotein composition presented elsewhere in this volume.

A. Measurements

The basic principles of spectrophotometry are applicable in the use of infrared absorption as a method of quantitative analysis (4,49). The fundamental physical measurement is that of light intensity — or, more appropriately in the infrared, radiant power. The power is supplied by the radiation source; a narrow wavelength interval is selected by the monochromator; and the emerging energy is measured by the thermocouple. The resultant electrical signal is proportional to the intensity I_o, which is to be compared with I_s, the corresponding energy measurement when an absorbing substance is placed in the radiation beam. The ratio of I_s to I_o is the transmittance, T, of the substance. (In double beam spectrophotometers, as described in an earlier section, I_s and I_o are measured in separate sample and reference beams.)

Since transmittance is exponentially related to the concentration of the absorbing substance, a useful function is the absorbance, A, defined as follows:

$$A = -\log_{10} T = \log_{10} \frac{I_o}{I_s}$$

It is equivalent to optical density.

For a given substance at a given (monochromatic) wavelength λ, the absorbance is determined by three factors: (1) c, the concentration of the substance; (2) b, the path length through it; (3) a_λ, its absorptivity at wavelength λ. The factors b and c define the number of molecules in the light path. Absorptivity (equivalent to extinction coefficient) is equal to the absorbance at unit concentration and unit path length and is an

intrinsic property of the substance. (The variation of absorptivity with wavelength is implicit in the spectral absorption curve.) The relationship of these factors is expressed as the well-known Beer-Lambert Law:

$$A_\lambda = \underline{a}_\lambda \underline{b}\underline{c}$$

For instrumental reasons it has not been generally possible in the infrared to establish absolute absorptivity values that can be used with confidence in all spectrophotometers. Furthermore, at the small path lengths used it is hazardous to rely on measured cell thicknesses. Rather it is common practice to carry out empirical calibrations using pure standard compounds in the same cell that is to be used subsequently for the analysis. If the working curve of absorbance vs. concentration is a straight line, the equation above can be reduced to

$$A_\lambda = \underline{k}_\lambda \underline{c}$$

where $\underline{k}_\lambda$, the empirically determined slope, represents a combination of $\underline{a}_\lambda$ and $\underline{b}$ for the particular cell.

A plot of A_λ vs. $\underline{c}$ can be non-linear for reasons which involve the relationship between band shape and spectral slit width. To avoid curvature from this cause, measurements should be made at band peaks rather than on steep sides of bands, and the spectral slit width should be kept narrower than the absorption band width. The latter requirement may be in conflict with the necessity for a wide enough slit width to provide adequate energy for measurement, but in a grating instrument with slit control this does not usually present any difficulty.

Another possible cause of non-linearity in the calibration of substances in solution is a change in band shape resulting from solute-solute interaction. Such interactions tend to increase with concentration, and they can be minimized

by working in a low concentration range. This in turn may require the use of relatively long path length cells. For most lipids that are soluble in CCl_4 or CS_2, concentrations less than 10 mg/ml in a 1.0 mm cell are satisfactory, and good approximations to linearity are usually obtained.

For quantitative measurements the use of dilute solutions has definite advantages over solid or undiluted liquid samples. If the amount of a lipid sample is about 2 mg or more the technique is simple and straightforward and does not require accessory equipment. The minimum volume of solvent needed for a 1.0 mm cell is about 0.5 ml, which is large enough so that solution volumes can be measured accurately. Constancy of path length is assured if all measurements are made in the same fixed thickness cell. Furthermore, a duplicate cell filled with pure solvent in the reference beam is probably the most effective type of background cancellation. Pure solvent in both cells represents a zero concentration point, for which the instrument may be adjusted to read 100% T (or an appropriate correction can be made). Thin cells ($\leq$ 0.1 mm) are not very suitable for solution analysis because higher concentrations are necessary and the correspondingly smaller volumes are less accurately measurable. There is also a greater "dilution" of the solvent in the sample cell, so that there can be a serious disparity with the amount of pure solvent in a matching reference cell. A variable-thickness reference cell is a possible means of correcting the solvent imbalance in that case.

Analysis in solution can be extended to the submilligram range by using microcells. Still longer path lengths ($>$ 1 mm) are desirable to increase sensitivity and minimize volume measurement problems. Solvent interference then becomes more likely, however.

If analysis in solution is precluded by insolubility, by solvent obscuration of the absorption band to be measured, or by too small sample size, the KBr pellet technique may be used. It is probably the only practicable technique for amounts smaller than about 100 μgm. For a given pellet die the thickness of the pellet formed is proportional to the weight of KBr. If the concentration is expressed on a weight/weight basis, the Beer's law expression

$$A_\lambda = \underline{a}_\lambda \underline{b}\ \underline{c}$$

becomes

$$A_\lambda = \underline{a}_\lambda (\underline{k}\ W)\left(\frac{\underline{w}}{W}\right)$$

where $\underline{k}$ is a constant relating thickness to weight, W is the weight of the pellet, and $\underline{w}$ is the weight of sample in the pellet. The expression further reduces to

$$A_\lambda = \underline{k}'\underline{w}$$

where $\underline{k}'$ is the product of $\underline{a}_\lambda$ and $\underline{k}$.
Thus the calibration can be established as absorbance vs. sample weight, independent of pellet weight, provided all of the sample is in the pellet. If there is loss of material in preparation of the pellet, or only a portion of the total (sample-KBr) mixture is used, it is necessary to know both the weight of the pellet and the weight of the total mixture in order to account for the difference. Since the KBr is usually weighed, and less than 1% of sample is added, the KBr weight may be used as the total mixture weight.

The use of an internal standard in KBr pellets was described by Wiberley *et al.* (50). In this procedure quantitation is based on the intensity ratio of an absorption band of the analyte and a band of a suitably chosen compound which is incorporated into the pellet in known amount. The requirements on

the internal standard are that its spectrum be relatively simple, so as not to have too many interfering bands, and that it have a well-isolated band of sufficient intensity to be suitable as the reference band. Substances that have been used are potassium thiocyanate, lead thiocyanate, and potassium ferricyanide. In all of these the nitrile absorption is the reference band. If the amount of the internal standard is kept constant we may write Beer's law expressions for the two independent bands and divide, obtaining

$$\frac{A_1}{A_2} = \frac{a_1 \, b \, c_1}{a_2 \, b \, c_2} = k \, c_1$$

where subscript one refers to the sample and subscript two the standard. The ratio a_1/a_2 is constant, b/b is unity, and together with c_2 they can be combined into the constant k.

Oil mulls and films, either liquid or solid, are unfavorable sampling forms for quantitative analysis. They are neither accurate nor reproducible in thickness, and in the case of films deposited by evaporation of solutions they are apt to be non-uniform as well. The internal standard method was proposed for mulls by Barnes, et al. (51); and in principle it is applicable to films, but its practical utility has evidently not been explored.

A method that is often useful for determining a minor component of a mixture is differential analysis. This is also applicable when the absorption band to be measured is overlapped by the band of another component. The procedure is essentially the solution procedure, except that as a reference pure solvent is replaced by a solution containing all the sample components other

than the one to be determined. The absorption of the "other" components is thereby balanced out in the two beams. The band to be measured becomes more distinct and the background is flattened out. The feasibility of this method will depend on the complexity of the mixture, and needs to be evaluated for a particular problem. Robinson (52) has shown that the optimum accuracy is attained when the concentration is adjusted to 37% transmittance without any compensation in the reference beam.

Whereas in solutions the transmittance of pure solvent establishes an I_o, or background level, KBr blanks are not reproducible enough to use as a reference for pelletized samples. The scattering background is much too variable from pellet to pellet, and especially from blank to sample. Under these circumstances it is necessary to estimate the I_o value by somewhat arbitrary empirical methods. One such method is commonly called the base-line method, and is illustrated in Fig. 15. It consists of connecting two suitably chosen points, one on each side of the absorption band being measured, by a straight line. The ordinate value of this line at the wavelength of the band peak is taken as I_o. The case illustrated shows a well separated absorption band, and the baseline is drawn tangent to the curve near the adjacent transmission maxima. It is intended to be an objectively constructed representation of what the curve might be if the absorption band were not there. An alternate method is the empirical ratio, shown in Fig. 15. Here I_o is taken as one of the transmission maxima adjacent to the absorption band. In the simple cases illustrated the baseline method might seem to be the more rational choice. However, the true value of I_o may lie somewhere between these two approximations and only trial and error will show which is better. The choice may be even less obvious when the spectrum is more complex, with multiple or overlapping bands in the region of interest.

The contribution of instrumental measurement error to the overall analytical error can be estimated if it is assumed that the (transmittance) scale error is constant over the whole scale, and applies equally to the 0%, 100%, and transmittance measurements. It was shown by Robinson (53) that a 1% scale reading error leads to a minimum concentration error of about 3.5% at a transmittance value of 37%. The error increases at higher and lower transmittance values, becoming 4% at about 20% T and 60% T, and 6% at about 10% T and 75% T. At still higher and lower transmittances the error curve rises quite steeply, and it is generally recognized that these extremes should be avoided if possible. The better classes of modern spectrophotometers should be capable of precision well within 1%. If this measurement is the limiting error, and transmittances are kept between 20% and 70%, analytical precision of 3% or less should be possible. Background estimation in the spectra of solids is obviously subject to greater error than solution measurements. This re-emphasizes the superiority of the solution mode.

The foregoing discussion has dealt mainly with the determination of a particular component by a single absorbance measurement. A simple example is the measurement of the amount of an isolated lipid or lipid class. In other cases, where more than one substance is present, the extraneous absorbances are considered as background and are accounted for by a suitably constructed base-line, or by a differential technique. For a mixture of only two components whose total amount is known, a working curve may be established for one component and the other determined by the difference.

To analyze for all $\underline{n}$ components of a mixture it is required that they be known and can be calibrated independently with pure standards. Essentially the method consists of measuring $\underline{n}$ absorbances at $\underline{n}$ appropriately chosen

spectral positions and inserting them in $\underline{n}$ linear equations:

$$C_1 = \underline{k}_{11}A_{\lambda_1} + \underline{k}_{12}A_{\lambda_2} \cdot \cdot \cdot \cdot \cdot \cdot + \underline{k}_{1n}A_{\lambda_n}$$

$$C_2 = \underline{k}_{21}A_{\lambda_1} + \underline{k}_{22}A_{\lambda_2} \cdot \cdot \cdot \cdot \cdot \cdot + \underline{k}_{2n}A_{\lambda_n}$$

$$\vdots$$

$$C_n = \underline{k}_{n1}A_{\lambda_1} + \underline{k}_{n2}A_{\lambda_2} \cdot \cdot \cdot \cdot \cdot \cdot \; \underline{k}_{nn}A_{\lambda_n}$$

where C_n = concentration of the nth component, A_{λ_n} is the measured absorbance at λ_n, and each $\underline{k}_{ij}$ is a constant. The values of $\underline{k}_{ij}$ are derived by matrix inversion of the $\underline{a}$'s in the set of equations:

$$A_{\lambda_1} = \underline{a}_{11}C_1 + \underline{a}_{12}C_2 \cdot \cdot \cdot \cdot \cdot \cdot \; \underline{a}_{1n}C_n$$

$$A_{\lambda_2} = \underline{a}_{21}C_1 + \underline{a}_{22}C_2 \cdot \cdot \cdot \cdot \cdot \cdot \; \underline{a}_{2n}C_n$$

$$\vdots$$

$$A_{\lambda_n} = \underline{a}_{n1}C_1 + \underline{a}_{n2}C_2 \cdot \cdot \cdot \cdot \cdot \cdot \; \underline{a}_{nn}C_n$$

Here $\underline{a}_{ij}$ is the calibration coefficient of the jth component at λ_i. This formulation is based on the assumptions: (1) that the individual calibration curves of all components at all wavelengths are linear; (2) that the individual absorbances are independent and additive. Except in unusual cases of interaction between components, the latter assumption is generally valid. The first assumption (Beer's Law) is tested in the course of calibration; and for most lipids in dilute solution the approximations to linearity are acceptable.

A further condition to be fulfilled is that for each component a wavelength (absorption band) can be chosen at which that component is the major absorber and all others absorb substantially less. Inability to meet this

condition precludes the use of this multicomponent method for many complex mixtures, although in favorable cases as many as ten components have been analyzed successfully. The problem of matrix inversion can now be handled easily by computers, although for just two components the arithmetic is very simple. Some useful two-component lipid analyses will be cited later. (A more extensive treatment of multicomponent analysis is given in Ref. 49, p. 403).

B. Analytical Methods for Blood Lipids.

Since infrared absorption measurements are made almost exclusively in non-aqueous media, the analysis of blood lipids requires a preliminary extraction. Methods of extraction are discussed in another chapter of this volume, and will only be touched on briefly here. In most instances a total lipid extract is obtained and subsequent fractionation of some sort is carried out. Procedures that are adequate to extract all of the lipids also carry along some nonlipid material, which should be removed as a first step of purification. For serum the method of Sperry and Brand (54) is generally satisfactory. The solvent used is chloroform-methanol (2:1), and the nonlipid material extracted is removed by a water wash. Nelson (31) has described a procedure for the extraction of red cells, also using chloroform-methanol as the solvent. In this case the subsequent removal of nonlipid contaminants is accomplished by a gel filtration technique, slightly modified from that of Siakotos, *et al*. (55). Other extraction procedures may be adopted in special circumstances, and in consideration of the particular lipids to be determined and the type of fractionation to be employed.

The amount of a total serum lipid extract — purified as described above — can be determined with an accuracy and reproducibility of about ± 3%, as compared with weight measurement. This is done by measuring the absorbance in CCl_4 solution at the CH_2 bending frequency, 6.8 μm (1465 cm^{-1}). Calibration of the various major components at this spectral position gives absorption coefficients which are all within a 10% range. Using an average value weighted according to a normal serum lipid composition results in closer agreement. Wide variations in fatty acid chain length or degree of unsaturation would affect the intensity of this band and cause larger errors. Presumably the C-H stretching band near 3.45 μm (2900 cm^{-1}) could be used in a similar way, although the corresponding calibrations have not been carried out. These bands would be more suitable for microanalysis, since they are more intense and also more free from solvent interference that may be encountered in longer path length cells.

Any available separation method may be used to isolate lipid classes or to simplify the lipid mixture to be analyzed. In practice, column and thin-layer chromatography are the most generally useful. These methods are also treated elsewhere in this volume, and will be described only minimally here as necessary. Better separations are usually possible by thin-layer chromatography, but the amounts are small enough to require microsampling techniques for infrared measurement, especially with the less abundant lipid species. Column chromatography can provide adequate sample sizes but for some lipid classes the separation problem is formidable.

For an isolated lipid class, the infrared analysis is straightforward. Taking account of possible spectral variations within the class, any absorption band — preferably a strong one — may be chosen for calibration and measurement. It should be noted for example, that in an ester class (e.g.,

cholesteryl esters) containing several fatty acids the carbonyl absorption is proportional to the concentration of C=O groups. Calibration on a molar concentration basis is therefore strictly valid, whereas for weight calibration an average molecular weight must be assumed. This is the same assumption that is commonly made in other methods of analysis, and it is not unreasonable for the fatty acid compositions normally encountered in blood lipids. It should be borne in mind however, in unusual situations such as the administration of high levels of medium chain fatty acids in the diet.

A method for determining the major serum lipids by a combination of column chromatography and infrared measurement was devised by Freeman, *et al.* (56). In view of the difficulty of isolating all of the lipid classes, they adopted a simplified, more manageable procedure in which only three fractions are obtained. Using small silicic acid columns (0.25 gm or 1.0 gm), the total lipid extract from 1.0 ml of serum is applied in hexane and eluted successively with hexane-chloroform (95:5), chloroform, and methanol. Each fraction is evaporated to dryness and redissolved in a known volume of CS_2 or CCl_4 for infrared measurement. Typical spectra are shown in Fig. 16. The first fraction contains only cholesteryl esters, which can be determined from the absorbance of the ester carbonyl band. The second fraction is a mixture of triglycerides, unesterified cholesterol, and unesterified fatty acids. Normally the amount of fatty acids is too small to measure and its contribution to the spectrum (5.85 μm, 1710 cm^{-1}) can be ignored. In this case the concentration of triglycerides is found directly from the ester carbonyl band. (Cholesterol does not absorb in the carbonyl region.) The absorbance is then measured at the cholesterol peak at 9.5 μm (1050 cm^{-1}) and corrected for the contribution of the already calculated amount of triglycerides. The

corrected absorbance value gives the cholesterol concentration. In cases where significant amounts of unesterified fatty acids are present, a two-component analysis based on the overlapping C=O bands is carried out, and a double correction is applied to the cholesterol peak measurement. This method has been used in lipolysis experiments to follow the production of free fatty acids (57,58).

The fraction eluted by methanol consists of the total mixture of phospholipids. Its spectrum resembles that of lecithin, the only readily observable difference being a slightly lower relative intensity of the ester band due to the presence of 20% sphingomyelin. Among the stronger infrared bands, the best choice for absorbance measurement is one of the doublet peaks at about 9.1 μm (1100 cm^{-1}) and 9.4 μm (1065 cm^{-1}). Absorptivities of the component phospholipids are nearly the same in this region, except that of phosphatidyl ethanolamine is slightly less at the shorter wavelength peak. If this were a major constituent the 9.4 μm peak would be preferable. The ester band at 5.76 μm (1737 cm^{-1}) can also be used, provided that the proportion of sphingomyelin is approximately constant in all samples. In the latter case the total serum phospholipid mixture is used as a calibration standard, whereas egg lecithin is a suitable reference material for the phosphate ester bands.

To the extent that further fractionation is possible, other individual lipids can be measured. For example, a more exacting chromatographic procedure such as that of Hirsch and Ahrens (59) might be used to obtain mono - and diglycerides. Phospholipids can be separated by the highly refined system of gradient elution chromatography developed by Nelson (60). In

simpler versions described earlier by Freeman (13) and Nelson and Freeman (61) only partial separations are achieved. Nevertheless the fractions containing lecithin and sphingomyelin together can be analyzed by the two-component method, using the ester and amide carbonyl bands at 5.76 μm (1737 cm^{-1}) and 6.1 μm (1640 cm^{-1}).

A method for determining triglycerides based on thin-layer chromatography and infrared spectrophotometry was devised by Krell and Hashim (62). They used adsorbent layers 1 mm thick in order to accommodate the amounts of lipid obtained from 1 ml of serum. After development of the plate, the adsorbent was scraped from triglyceride spot areas and the triglycerides were eluted from it. Spectra were run in CS_2 solutions, and absorbance was measured at the ester carbonyl peak, using the baseline method. Under these conditions some curvature was noted in the absorbance-concentration plot, and this could be taken into account either by reading directly from the curve or by a mathematical correction to the measured absorbance value. Presumably this method could be used for other components as well.

Triglycerides and cholesteryl esters in serum can be determined simultaneously by the two-component method (48). This analysis requires the removal of the phospholipids from the total extracted lipid, and infrared measurements on the remaining mixture. Such a separation is also required in the chemical triglyceride methods (63) currently in use. It may be accomplished by batch adsorption on silicic acid from chloroform or chloroform-acetone solution, or by adsorption on a column. Another alternative procedure is to combine the extraction and adsorption in a single step as described by Mendelsohn and Antonis (64). The latter yields comparable results for

triglycerides, and only slightly less reliable results for cholesterol esters. However achieved, the mixture after phospholipid removal contains cholesteryl esters and triglycerides as the major components, with lesser amounts of cholesterol and unesterified fatty acids. Cholesterol has negligible absorption in the carbonyl region, and with few exceptions fatty acids are present in serum in small enough concentrations so that their contribution to the ester peak absorbances can be ignored. The overlapping carbonyl bands of cholesteryl esters and triglycerides are shown in Fig. 17. Measurements are made at the two peak positions. Where one component is the major absorber, there is a contribution to the absorbance from the side of the band of the other component. Thus not only is good resolving power needed, but also good reproducibility of frequency settings. A grating instrument is preferred, but if other requirements are met it may be possible to use a sodium chloride prism and very narrow slits. Absorbances are not measured on a recorded scan, but at stationary points set manually. Calculations are made by a two-component matrix as described earlier. The prime virtue of this method is operational simplicity, and it is also nondestructive. By using a microcell it can be adapted to smaller quantities of serum, down to 0.1 ml.

A prototype instrument has been described for carrying out the triglyceride-cholesteryl ester analysis in a semiautomatic manner (65). This device incorporates a small single-beam grating spectrophotometer, which must be purged with dry air to minimize water vapor absorption. It is equipped with a motor-actuated grating positioner, programmed to be set alternately at the two measurement frequencies. The transmittance measurements, represented by voltages, are first converted electronically to absorbances.

These are multiplied by the predetermined constants of the matrix equations, and the appropriate terms combined. Calculated results are displayed on a digital voltmeter and also printed on tape.

The analysis of total unfractionated lipid extract from serum by infrared spectrophotometry has been undertaken (13), but with limited success. Examination of the spectrum of the total lipids (Fig. 18) with reference to the component spectra leads to the conclusion that the characteristic absorption bands for the various lipid classes are not sufficiently distinctive to permit a multicomponent analysis of the mixture in terms of those classes. For example, the differentiation of triglycerides and cholesteryl esters in the presence of phospholipids is difficult if not impossible. However, instead of the actual classes, components may be defined on the basis of significant absorption bands related to chemical groups. Thus the carbonyl band might be used for measuring total esterified fatty acids (TEFA), one of the phosphate ester bands for the phopholipids (PL) and the methyl group band for total cholesterol (TC). Since phospholipids contain esterified fatty acids, these three arbitrary components are not independent. It is necessary to define an artificial phospholipid as the residue obtained from the total phospholipids by removal of the esterified fatty acids. If it is assumed that the residue is a constant fraction of the total, the conversion can be made by a simple numerical factor. Calling this defined component residual phospholipid (RPL), we have three things which add up to the total lipid, i.e., TL = TEFA + TC + RPL. (Glycerol and free fatty acids are ignored.) Since each is associated with a different characteristic absorption band, it should

be possible to set up a three-component analysis based on the usual equations:

$$A_{\lambda_1} = \underline{a}_{11}C_{TEFA} + \underline{a}_{12}C_{RPL} + \underline{a}_{13}C_{TC}$$

$$A_{\lambda_2} = \underline{a}_{21}C_{TEFA} + \underline{a}_{22}C_{RPL} + \underline{a}_{23}C_{TC}$$

$$A_{\lambda_3} = \underline{a}_{31}C_{TEFA} + \underline{a}_{32}C_{RPL} + \underline{a}_{33}C_{TC}$$

where A = absorbance

C = concentration

λ_1 = 5.76 μm (carbonyl)

λ_2 = 9.1 μm (phosphate ester)

λ_3 = 7.25 μm (methyl)

$\underline{a}_{ij}$ = calibration coefficient of component j at wavelength $\underline{i}$.

To proceed according to this formulation it is necessary to evaluate the $\underline{a}_{ij}$'s, and here some difficulties are encountered. Whereas normally these would be calibration coefficients determined for specific compounds, in this instance this is only true for cholesterol and all others are derived. For example, a coefficient for TEFA at λ_3 may be determined from separate calibrations of cholesteryl ester (CE) and unesterified cholesterol (UC). The assumption is made that the ester absorptivity is the weighted sum of its component absorptivities, i.e.,

$$\underline{a}_{CE} = \underline{a}_{EFA} \times (\text{wt. fraction EFA}) + \underline{a}_{UC} \times (\text{wt. fraction UC})$$

The value of $\underline{a}_{EFA}$ ($\underline{a}_{31}$) so obtained is in good agreement with the value derived from triglyceride calibration. For some of the other coefficients obtained in a similar way however, there is greater variability, so that

the use of average values leads to errors at extremes of sample composition.

At λ_1 only the ester groups absorb. For the various esters the absorbance per fatty acid (concentration) can be determined simply from the molecular weight ratio of ester to fatty acid. Since the carbonyl peak positions of cholesteryl esters, triglycerides, and phospholipids are not precisely the same, λ_1 has to be carefully selected so that $\underline{a}_{11}$ is as nearly equal as possible in all three classes. As may be seen in Fig.22 this point corresponds approximately to the phospholipid peak. Since cholesterol does not absorb in the carbonyl region, and residual phospholipid has been defined to exclude fatty ester groups, both $\underline{a}_{12}$ and $\underline{a}_{13}$ may be set equal to zero. The first equation is thereby reduced to a single term, and TEFA can be calculated independently from a single absorbance measurement. This TEFA concentration can then be substituted in the other two equations, reducing each of them by one term. There remains then a 2 x 2 matrix, but the absorptivity of cholesterol at λ_2 is very small and may be neglected. This eliminates another term from the second equation. The RPL concentration can be calculated from the remaining term and substituted in the third equation, which is then simply solved for cholesterol concentration. Thus the zero absorptivity values permit the matrix calculation to be avoided altogether.

Operationally this method is extremely simple. Spectrophotometry is carried out directly on the purified serum lipid extract dissolved in a measured volume of CCl_4. The information obtained is essentially that from separate chemical determinations of total cholesterol, total esterified fatty acids, and lipid phosphorous. (A total lipid measurement may also be made at the same time.) The method has good precision, and it is accurate to about

5 - 10% in the normal range of serum lipid compositions, for which its inherent assumptions and empirical averages are acceptable. However it cannot be regarded as generally useful for a wide range of lipid compositions. Furthermore it does not provide a good determination of triglycerides, since it would depend on calculations of difference between total fatty acids and the fatty acids of cholesteryl esters and phospholipid, just as in the older indirect chemical methods.

An infrared method has been used in conjunction with ultracentrifugal fractionation to measure chylomicrons in serum at the microgram level (66). The isolation of the chylomicron-containing fraction is carried out by flotation in a sodium chloride density gradient solution, using a swinging-bucket rotor. After centrifugation the top milliliter in the tube contains the serum lipoproteins designated as $S_f > 400$, which are made up principally of the chylomicrons. Since the solution contains a known concentration of sodium chloride, it is possible to lyophilize an aliquot and prepare a pellet of NaCl in the manner that has been described for KBr. A microdie is used, forming a pellet 1.5 mm in diameter and containing about 5 mg of NaCl. For this size pellet a beam condenser is used. The amount of chylomicrons that can be measured (without dilution) is 1 - 40 μgm. Some curvature of the calibration curve is indicated above about 25 μgm. Absorbance is measured at the triglyceride peak, using a baseline method as shown in Fig.19. Calibration is carried out with dilutions of a concentrated chylomicron preparation, in which the triglyceride content is known from independent analysis.

In addition to the determination of the lipid classes, there are some infrared analyses related to fatty acid characteristics. Since quantitation

of individual fatty acids is done largely by gas chromatography, the infrared methods are supplementary, or they are applied to mixed lipids or lipid classes for special purposes. Perhaps most significant are those related to unsaturation.

On the assumption that all of the double bonds in the fatty acids of blood lipids are of the cis unconjugated type, total unsaturation can be measured at either of two absorption bands. One of these bands is the (C=)C-H stretching absorption at 3.3 μm (3020 cm^{-1}). Sinclair et al. (28) proposed a method for the fatty acids, suitable also for triglycerides and methyl esters and presumably other classes as well. In this method the ratio of the 3020 cm^{-1} band intensity to the difference between it and the CH_2 stretching intensity at 3.4 μm (2920 cm^{-1}) is plotted as a (linear) function of the number of double bonds per molecule. Since a ratio is used the total concentration need not be known. An alternative is to plot the 3020 cm^{-1} band intensity against degree of unsaturation, using a fixed ester concentration. With a sodium chloride prism instrument this band is not well enough resolved for good measurement, but any higher degree of resolution is satisfactory. A fluorite prism was used by Sinclair et al., and a grating is clearly adequate.

The other absorption band at which cis unsaturation can be measured is at 14.2 μm (700 cm^{-1}). Here resolution is not a problem. This band is overlapped by the CH_2 chain rocking absorption at 13.9 μm (720 cm^{-1}), but it can be calibrated either directly or by a differential method (14). In the latter procedure triglycerides were used with the sample being measured against tripalmitin (both in CS_2 at the same concentration - 40 mg/ml in a 0.9 mm cell). Calibration spectra are shown in Fig.20.. At low degrees of unsaturation this

band (as well as the 3020 cm^{-1} band) is not very strong, so that relatively high concentrations are needed.

The determination of unconjugated trans isomers of unsaturated fatty acids is perhaps the best known infrared method in the chemistry of fats and oils, mainly because of its importance in connection with the hydrogenation process, but also because gas chromatographic separation of the isomers is difficult. The method was first described by Shreve *et al.* (67) and numerous variations of it have been published. One procedure has been adopted as Standard Method Cd 14-61 of the American Oil Chemists' Society. The levels of trans isomer are low in blood lipids, and they are believed to be exclusively of dietary origin. No definitive study of them has been made, although an adaptation of the method and a few measurements have been reported (68). In this procedure the serum lipids are methanolyzed to obtain methyl esters. The ester spectra are recorded in CS_2 solution at a concentration of 15 mg/ml in a 3.5 mm microcell. Curves in the spectral interval 9.5 - 10.5 μm (1050 - 950 cm^{-1}) are shown in Fig.21 for different percentages of methyl elaidate in methyl oleate. Band intensities are measured by the baseline method, in which the 100% oleate curve approximates the background. By using the same solution for spectral measurement at 14.2 μm (700 cm^{-1}) the cis double bonds are determined in the same sample, thus giving total unsaturation.

It is possible to estimate the average chain length in mixed saturated fatty acids from the ratio of C-H to C=O absorptions (69), but this method is of little utility in the blood lipid system. Also the estimation of the degree of branching from the CH_3 symmetrical bending band (34) is not likely to be applicable, since branched acids — if they occur in blood lipids — are at

most trace components.

C. Use of Computers

Specialized computing devices have been used for a long time in conjunction with infrared spectrophotometry. A particular application has been in connection with multicomponent analyses in which large matrices are required. These can be handled by analog computers designed for this purpose, using absorbances measured in the usual way as input data. The automatic two-component analyzer mentioned in the previous section contains simple computing circuitry of this type.

In recent years, with the increasing availability of general purpose large-memory digital computers, various aspects of spectrometric data handling can be treated with greater facility. Some of these aspects are related to the techniques of correcting instrument performance, refining data and improving spectral quality, e.g., background and frequency corrections, peak location, and curve smoothing.

Applications of this type, as well as computer methods for spectrum identification and qualitative structural diagnosis, have been reviewed by Crisler (70). Other applications are more specifically related to analysis, and in addition to straight arithmetic and matrix computation these include the determination of integrated band intensities, resolution of overlapping bands, and component band summation.

For use by the computer, the output of the spectrometer is first converted to digital form and stored on punched cards, magnetic tape, or punched paper tape. The spectrum is thereby represented as a sequence of transmittance or absorbance values measured at closely spaced intervals over the whole range.

A limited spectral range may be selected for a specific purpose; and all or part of the spectrum can be plotted on any desired scale from the stored data.

Computer methods have been explored in connection with some infrared analyses of blood lipids (14). One approach is to use integrated band intensities, which are often more reliable than peak absorbances. A determination for which this method can be used is that of total esterified fatty acids (TEFA), based on measurement of the ester carbonyl band. Although in the spectrum of a serum lipid extract this band has a single peak, it is a composite of the absorptions of cholesteryl esters, triglycerides, and phospholipids. The individual bands of these ester classes are shown on an expanded scale in Fig. 22. Concentrations, have been adjusted to equivalent fatty acid concentration for all types. Although a curve is included for unesterified fatty acid at the same level, the amount of this component in serum is normally less than 10% of the total fatty acids. In the method described in the previous section, TEFA were estimated from an absorbance measured at approximately the lecithin peak position. At that point the absorbance per fatty acid is nearly the same for all three components. However the measurement is off the peaks of both triglyceride and cholesteryl ester, and therefore highly dependent on the reproducibility of the frequency setting. It is particularly subject to errors at very high or very low triglyceride content. Use of integrated band intensities avoids this difficulty and should be more reliable.

The integrated intensities used here are not absolute, but are empirically measured band areas. Over the interval from 1690 - 1790 cm^{-1} a transmittance value is recorded every 0.5 cm^{-1}. These values are converted to absorbances,

which are then summed and multiplied by 0.5 to obtain the area. This calculation is expressed in the following equation:

$$\text{Band area} = B = \int_{\nu_1}^{\nu_2} A d\nu = \sum_{1690}^{1790} A \Delta\nu = 0.5 \sum_{1690}^{1790} A$$

The band area data for absorption by various lipids in this spectral interval are summarized in Table III. In this table the ranges of values are given corresponding to indicated ranges of concentration. B/C does not vary appreciably in these ranges, indicating adherence to Beer's Law. The constancy of B/C_{FA} over all four ester classes verifies that the band area per ester group is the same for all of the ester types and is therefore valid for mixtures of them. In known mixtures which do not include unesterified fatty acids the accuracy of the determination has been estimated to be about ± 2%. When free fatty acids are present the error increases with their amount and reaches about 10% when the fatty acids comprise 10% of the total. This error can be reduced to less than 5% by choosing a narrower spectral interval (1770-1710 cm^{-1}) which excludes a large part of the fatty acid absorption band without seriously affecting the constancy of the ester band area.

This procedure has also been applied to the measurement of total lipid, using the CH_2 bending band. It has been noted in the previous section that the 6.8 μm (1465 cm^{-1}) peak can be used successfully for this determination in serum lipids. By the use of band areas it appears that the measurement is valid over a wider range of sample compositions. Fig.23 shows the band patterns for representative lipids, and two possible integration intervals are indicated. In TableIV the areas are compared with peak absorbances for various lipid classes, some of which include fatty acids of differing unsaturation. The effect of unsaturation is clearly revealed in these data. The overall

constancy with respect to both lipid classes and unsaturation is greatest in the integrated absorptivities of the narrower interval (1470 - 1420 cm^{-1}). This area therefore should be least affected by sample composition.

Another way of using the computer is to match the absorption curve of a mixture to the summation of the individual component curves, as illustrated in Fig. 24. Here the dotted curve was regenerated from the spectrum recorded for a known mixture of triolein, cholesteryl oleate, and oleic acid. These represent the nonphospholipid components from serum which can be measured in this spectral interval. The upper solid curve is the sum at every abscissa point of the ordinates of the three lower curves, which are spectra of the standards. Component concentrations indicated for the standards are those determined by the computer by a least-squares curve fitting method, such that the summation curve is the best possible fit to the recorded curve. The average error for triglycerides and cholesteryl esters in a series of such mixtures was about 3%. Since the amount of fatty acid is very small the relative error for this component is high - about 20% at the level in the example used here. Although an accurate determination of free fatty acids is not to be expected under these conditions, a useful analysis for them should be possible by careful refinement of this method.

Two possibilities may be suggested for using a computer to assist in the analysis of total unfractionated serum lipids. One is an elaboration of the method outlined in the previous section, wherein total lipids, total cholesterol, esterified fatty acids, and total phospholipids are determined. Modifications to that procedure might include replacement of peak absorbances by band area measurements and use of an iterative calculation with adjustable

constants. The second approach is to analyze the carbonyl band for the three major ester components by the least-squares curve fitting procedure mentioned above. A preliminary investigation of this method has been reported (14), but more development work is needed in these areas. Since neither method determines all components some combination of them may be necessary for a total analysis.

There is a clear trend toward the use of computers in conjunction with several aspects of infrared spectroscopy, as there is indeed with nearly all analytical systems. Although the automation of relatively simple analyses is a worthwhile function, it is often the capability of performing more sophisticated or complex analyses that makes the computer attractive. In this respect blood lipids are still a challenge. As instrumentation develops, infrared spectrophotometers will become more adaptable to direct interfacing with computers, thereby eliminating the intermediate stage of recording data on tape. It seems probable that in the future most infrared analyses - possibly including blood lipid analyses - will be "on line" with the computer.

FOOTNOTES

[1] 1 μm = 1 micrometer = 10^{-6} meter = 10^{-3} mm (formerly μ, micron).

[2] 1 cm^{-1} = 1 wave per centimeter (1 μm = 10000 cm^{-1}).

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REFERENCES

1. Potts, W. J., Jr., Chemical Infrared Spectroscopy, Vol. 1, Techniques, John Wiley and Sons, Inc., New York, 1963.

2. Rao, C. N. R., Chemical Applications of Infrared Spectroscopy, Academic Press, New York, 1964.

3. Meloan, C. E., Elementary Infrared Spectroscopy, Macmillan, New York, 1963.

4. Szymanski, H. A., Theory and Practice of Infrared Spectroscopy, Plenum Press, New York, 1964.

5. Colthup, N. B., L. H. Daly, and S. E. Wiberly, Introduction to Infrared and Raman Spectroscopy, Academic Press, New York, 1964.

6. Colthup, N. B., J. Opt. Soc. Am., 40, 397 (1950).

7. Bellamy, L. J., The Infrared Spectra of Complex Molecules, John Wiley and Sons, Inc., New York, 1958.

8. Bellamy, L. J., Advances in Group Frequencies, Methuen, London, 1968.

9. Chapman, D., The Structure of Lipids, John Wiley and Sons, Inc., New York, 1965.

10. O'Connor, R. T., J. Am. Oil Chem. Soc., 33, 1 (1956).

11. Schwarz, H. P., L. Dreisbach, R. Childs, and S. V. Mastrangelo, N. Y Acad. Sci., 69, 116 (1957).

12. Schwarz, H. P., in Advances in Clinical Chemistry, H. Sobotka and C. P. Stewart, Eds., Academic Press, New York, Vol. 3, 1960, pp. 1-34

13. Freeman, N. K., N. Y. Acad. Sci. 69, 131 (1957).

14. Freeman, N. K., J. Am. Oil Chem. Soc. 45, 798 (1968).

15. Holman, R. T., and P. R. Edmondson, Anal. Chem. 28, 1533 (1956).

16. Holman, R. T., S. Ener, and P. R. Edmondson, Arch. Biochem. Biophys. 80, 72 (1959).

17. Tarpley, W., and C. Vitiello, Anal. Chem. 24, 315 (1952).

18. Fridmann, S. A., in Progress in Infrared Spectroscopy, H. A. Szymanski, Ed., Plenum Press, New York, Vol. 3, 1967, pp. 1-23.

19. Mason, W. B., Microchem. J., Symp. Ser. 1, 293 (1961). Also Sparagana, M., and W. B. Mason, Steroids, 2, 245 (1963).

20. Leggon, H. W., Anal. Chem. 33, 1295 (1961).

21. Rouser, G., G. Kritchevsky, M. Whatley, and C. F. Baxter, Lipids 1, 107 (1966).

22. McCoy, R. N., and E. C. Fiebig, Anal. Chem. 37, 593 (1965).

23. Blinn, R. C., J. Assoc. Off. Ag. Chem. 46, 952 (1963).

24. Jones, R. N., A. F. McKay, and R. G. Sinclair, J. Am. Chem. Soc. 74, 2575 (1952).

25. Meiklejohn, R. A., R. J. Meyer, S. M. Aronovic, H. A. Schuette, and V. W. Meloch, Anal. Chem. 29, 329 (1957).

26. Wolff, I. A., and T. K. Miwa, J. Am. Oil Chem. Soc. 42, 208 (1965).

27. Labarrère, J. A., J. R. Chipault, and W. O. Lundberg. Anal. Chem. 30, 1466 (1958).

28. Sinclair, R. G., A. F. McKay, G. S. Myers, and R. N. Jones, J. Am. Chem. Soc. 74, 2578 (1952).

29. Rouser, G., G. Kritchevsky, D. Heller, and E. Lieber, J. Am. Oil Chem. Soc. 40, 425 (1963).

30. Nelson, G. J., Lipids 2, 323 (1967).

31. Nelson, G. J., Biochem. Biophys. Acta, 144, 221 (1967).

32. Nelson, G. J., Lipids 3, 104 (1968).

33. Kimura, Y., and Y. Nagai, Biochem. J. 77, 3 (1960).

34. Freeman, N. K., J. Am. Chem. Soc. 74, 2523 (1952).

35. Fischmeister, I., Arkiv. f. Kemi. 20, 353 (1962).

36. Fischmeister, I., Arkiv. f. Kemi. 20, 385 (1962).

37. Freeman, N. K., F. T. Lindgren, Y. C. Ng, and A. V. Nichols, J. Biol. Chem. 203, 293 (1953).

38. Ambrose, E. J., and A. Elliott, Proc. Roy. Soc. A, 208, 75 (1951).

39. Miyazawa, T., and E. R. Blout, J. Am. Chem. Soc. 83, 712 (1961).

40. Susi, H., S. N. Timasheff, and L. Stevens, J. Biol. Chem. 242, 5460 (1967).

41. Timasheff, S. N., H. Susi, and L. Stevens, J. Biol. Chem. 242, 5467 (1967).

42. Gotto, A. M., R. I. Levy, A. S. Rosenthal, M. E. Birnbaumer, and D. S. Fredrickson, Biochem. Biophys. Res. Comm. 31, 699 (1968).

43. Scanu, A., and J. L. Granda, in Progress in Biochemical Pharmacology, C. J. Miras, A. N. Howard, and R. Paoletti, Eds., S. Karger, Basel, Vol. 4, 1968, pp. 153-158.

44. Maddy, A. H., and B. R. Malcolm, Science 150, 1616 (1965).

45. Chapman, D., V. B. Kamat, and R. J. Levene, Science 160, 314 (1968).

46. Freeman, N. K., F. T. Lindgren, and A. V. Nichols, in Progress in the Chemistry of Fats and Other Lipids, R. T. Holman, W. O. Lundberg, and T. Malkin, Eds., Pergamon, London, Vol. 6, 1963, 228-233.

47. Ewing, A. M., N. K. Freeman, and F. T. Lindgren, in Advances in Lipid Research, R. Paoletti and D. Kritchevsky, Eds., Academic Press, New York, Vol. 3, 1965, pp. 50-51.

48. Freeman, N. K., J. Lipid Res. 5, 236 (1964).

49. Bauman, R. P., Absorption Spectroscopy, John Wiley & Sons, Inc., New York 1962, Chapter 9.

50. Wiberley, S. E., J. W. Sprague, and J. E. Campbell, Anal. Chem. 29, 210 (1957).

51. Barnes, R. B., R. C. Gore, E. F. Williams, S. G. Linsley, and E. M. Peterson, Ind. Eng. Chem., Anal. Ed., 19, 620 (1947).

52. Robinson, D. Z., Anal. Chem. 24, 619 (1952).

53. Robinson, D. Z., Anal. Chem. 23, 273 (1951).

54. Sperry, W. M., and F. C. Brand, J. Biol. Chem. 213, 69 (1955).

55. Siakotos, A. N., and G. Rouser, J. Am. Oil Chem. Soc. 42, 913 (1965).

56. Freeman, N. K., F. T. Lindgren, Y. C. Ng., and A. V. Nichols, J. Biol. Chem. 227, 449 (1957).

57. Shore, B., A. V. Nichols, and N. K. Freeman, Proc. Soc. Exp. Biol. Med. 83, 216 (1953).

58. Young, W., and N. K. Freeman, Proc. Soc. Exp. Biol. Med. 90, 463 (1955).

59. Hirsch, J., and E. H. Ahrens, Jr., J. Biol. Chem. 233, 311 (1958).

60. Nelson, G. J., J. Am. Oil Chem. Soc. 44, 86 (1967).

61. Nelson, G. J., and N. K. Freeman, J. Biol. Chem. 234, 1375 (1959).

62. Krell, K., and S. A. Hashim. J. Lipid Res. 4, 407 (1963).

63. Van Handel, E., and D. B. Zilversmit, J. Lab. Clin. Med. 50, 152 (1957).

64. Mendelsohn, D., and A. Antonis, J. Lipid Res. 2, 45 (1961).

65. Freeman, N. K., E. Lampo, and A. A. Windsor, J. Am. Oil Chem. Soc. 44, 1 (1967).

66. Hatch, F. T., N. K. Freeman, L. C. Jensen, G. R. Stevens, and F. T. Lindgren, Lipids 2, 183 (1967).

67 Shreve, O. D., M. R. Heether, H. B. Knight, and D. Swern, Anal. Chem. 22, 1261 (1950).

68. Freeman, N. K., Semiannual Report, Donner Laboratory, Lawrence Radiation Laboratory, UCRL-9897, 1961, p. 96.

69. Keeney, P. G., J. Am. Oil Chem. Soc. 39, 304 (1962).

70. Crisler, R. O., J. Am. Oil Chem. Soc. 45, 818 (1968).

TABLE I. ESTER AND CARBOXYL GROUP FREQUENCIES OF LIPID CLASSES ($IN\ CCl_4$ SOLUTION)

	C=O		C-O[1]	
	λ(μm)	(cm^{-1})	λ(μm)	(cm^{-1})
Triglycerides	5.73	1745	8.6	1160
Phospholipids (Esters)	5.76	1737	8.55-8.6	1165-1170
Cholesteryl Esters	5.78	1731	8.55	1170
Methyl Esters	5.74	1741	8.55	1170
Fatty Acids	5.85	1710	7.8	1280[2]

[1] Only the principal band of the C-O-C group is given. The second is not well identified.

[2] In fatty acids the C-O vibration is coupled with O-H bending, giving rise to a second band at about 7.1 μm (1410 cm^{-1}).

TABLE II. LIPID COMPOSITION OF PRINCIPAL SERUM LIPOPROTEIN CLASSES, BY INFRARED ANALYSIS

(Average % of Total Lipid ± Std. dev.[1])

Lipoprotein	Triglycerides	Cholesteryl Esters	Phospholipids	Unesterified Cholesterol[2]
VLDL (males)	60.4 ± 6.6	17.2 ± 3.7	19.0 ± 3.5	3.4 ± 0.7
" (females)	58.1 ± 4.5	20.6 ± 3.2	17.1 ± 5.0	4.2 ± 0.7
LDL (males)	9.4 ± 2.4	55.1 ± 1.5	24.4 ± 2.3	11.0 ± 0.3
" (females)	9.3 ± 2.3	53.4 ± 1.5	26.6 ± 1.8	10.6 ± 0.3
HDL (males)	8.8 ± 2.3	42.7 ± 2.2	40.1 ± 2.4	8.4 ± 0.4
" (females)	6.8 ± 2.1	41.8 ± 2.3	43.1 ± 1.7	8.3 ± 0.4

[1] Analyses of ultracentrifugal preparations from sera of 16 normal males and 16 normal females. Values have been adjusted proportionally to total 100% of extracted lipid weight.

[2] Not measured. (Calculated as cholesteryl esters x 0.20.)

TABLE III. CARBONYL BAND AREAS FOR REPRESENTATIVE LIPIDS (1690 - 1790 cm^{-1})

Compound	C (mg/ml)	B, Band Area	B/C	B/C_{FA} [1]
Triolein	0.89 - 3.54	3.72 - 14.51	4.16 - 4.09	4.35 - 4.28
Cholesteryl Oleate	1.94 - 7.75	3.70 - 14.61	1.91 - 1.83	4.42 - 4.22
Lecithin (Egg)	2.38 - 5.96	7.71 - 19.16	3.24 - 3.18	4.44 - 4.38
Methyl Oleate	0.78 - 5.29	3.20 - 21.50	4.16 - 4.06	4.37 - 4.26
Oleic Acid	0.75 - 3.04	4.23 - 17.28	5.71 - 5.63	5.71 - 5.63
Cholesterol	2.02 - 9.09	0.13 - 0.48	0.063- 0.053	———

[1] C_{FA} = Conc. of fatty acid (mg/ml)

(From Ref. 14.)

TABLE IV. ABSORPTIVITIES OF VARIOUS LIPIDS IN THE CH_2 BENDING BAND

Compound	α,[1] Peak	β,[2] 1490 - 1390 cm^{-1}	β, 1470 - 1420 cm^{-1}
Tripalmitin	.0515	1.68	1.31
Triolein	.0443	1.64	1.27
Trilinolein	.0380	1.61	1.19
Cholesterol Palmitate	.0509	1.49	1.25
Cholesteryl Oleate	.0476	1.50	1.24
Cholesteryl Linoleate	.0450	1.46	1.19
Cholesterol	.0446	1.38	1.19
Lecithin (Egg)	.0424	2.08	1.31
Diolein	.0428	1.77	1.30
Monoolein	.0404	1.85	1.26

[1] α = Absorbance per mg/ml

[2] β = Band area per mg/ml

(From Ref. 14.)

FIGURE CAPTIONS

Fig. 1. Infrared spectra of solvents (Reagent Grade) in 1.0 mm cells. Broken lines indicate absorption bands of ethanol (as normally present in $CHCl_3$ as a preservative).

Fig. 2. Infrared spectrum of white mineral oil (Nujol). Capillary film between NaCl plates.

Fig. 3. Infrared spectra: Palmitic Acid (Hormel) 5 mg/ml and 15 mg/ml.
Oleic Acid (Applied Science) $\sim$ 0.01 mm.
Linoleic Acid (Hormel) $\sim$ 0.01 mm.

Fig. 4. Infrared spectra: Palmitic Acid (Hormel), solid.
Methyl Palmitate (redistilled) 7 mg/ml, 1.0 mm cell.
Methyl Elaidate (Supelco) $\sim$ 0.01 mm.

Fig. 5. Infrared spectra: Methyl Oleate (Hormel) $\sim$ 0.01 mm.
Methyl Linoleate (Hormel) $\sim$ 0.02 mm.
Methyl Arachidonate (Hormel) $\sim$ 0.01 mm.

Fig. 6. Infrared spectra: Tripalmitin (Hormel) 7 mg/ml, 1.0 mm cell.
Tripalmitin (Hormel), solid.
Trilinolein (Hormel) $\sim$ 0.01 mm.

Fig. 7. Infrared spectra: Triolein (Hormel) $\sim$ 0.02 mm.
Diolein (Hormel) $\sim$ 0.02 mm.
α-Monoolein (Supelco) $\sim$ 0.02 mm.

Fig. 8. Infrared spectra: Cholesteryl Palmitate (Supelco) 10 mg/ml, 1.0 mm cell.
Cholesteryl Palmitate (Supelco), solid.
Cholesteryl Oleate (Supelco) 10 mg/ml, 1.0 mm cell.

Fig. 9. Infrared spectra: Cholesterol (Hormel) 10 mg/ml, 1.0 mm cell.
Cholesterol (Hormel), solid.
Cerebrosides (bovine, Supelco), solid.

Fig. 10 Infrared spectra: Lecithin (egg, Analabs) ~ 0.02 mm.
Dipalmitoyl Lecithin (Supelco), solid.
Lysolecithin (Supelco) ~ 0.01 mm.

Fig. 11 Infrared spectra: Sphingomyelin (bovine, Applied Science) ~ 0.02 mm.
Phosphatidyl Ethanolamine (Applied Science) ~ 0.01 mm.
Plasmalogen (Ethanolamine) (white matter, Supelco) < 0.01 mm.

Fig. 12 Infrared spectra: Phosphatidyl Serine (Supelco), solid.
Phosphatidyl Inositol (Supelco), solid.
Cardiolipin (Applied Science) ~ 0.01 mm.

Fig. 13 Infrared spectra of serum lipoproteins. Dried films on AgCl Plates.

Fig. 14 Infrared spectra of egg lipoprotein and some of its components. A, (a) moist film; (b) dried film; (c) film after extraction with acetone; (d) film after extraction with 1:1 alcohol-chloroform. B and C are CS_2 solutions. (From Ref. 37.)

Fig. 15 Methods for estimation of I_o. Upper: baseline. Lower: empirical ratio. (From Ref. 3.)

Fig. 16 Infrared spectra (CS_2 solutions) of serum lipid fractions obtained by chromatography on silicic acid. A, cholesteryl esters (broken curve is solvent background). B, Glycerides, fatty acids, cholesterol (two examples: broken curve, no fatty acids; solid curve, fatty acids present). C, phospholipids. Small letters designate bands used for measurements. (From Ref. 56.)

Fig. 17 Carbonyl absorption bands of triolein and cholesteryl oleate. Solutions in CCl_4: triolein, 3.11 mg/ml; cholesteryl oleate, 7.16 mg/ml. Cell thickness, 1.0 mm. Frequency scale expanded to 5 times normal. (From Ref. 48.)

Fig. 18 Infrared spectrum of a total lipid extract from serum. 10 mg/ml in CCl_4. Cell thickness, 0.9 mm. (From Ref. 13.)

Fig. 19 Infrared absorption bands of triglycerides (1745 cm^{-1}) and residual water (1640 cm^{-1}) in NaCl pellets (1.5 mm dia.) made from lyophilized chylomicron preparations. A baseline for the solid curve has been sketched in (dotted curve). (From Ref. 66.)

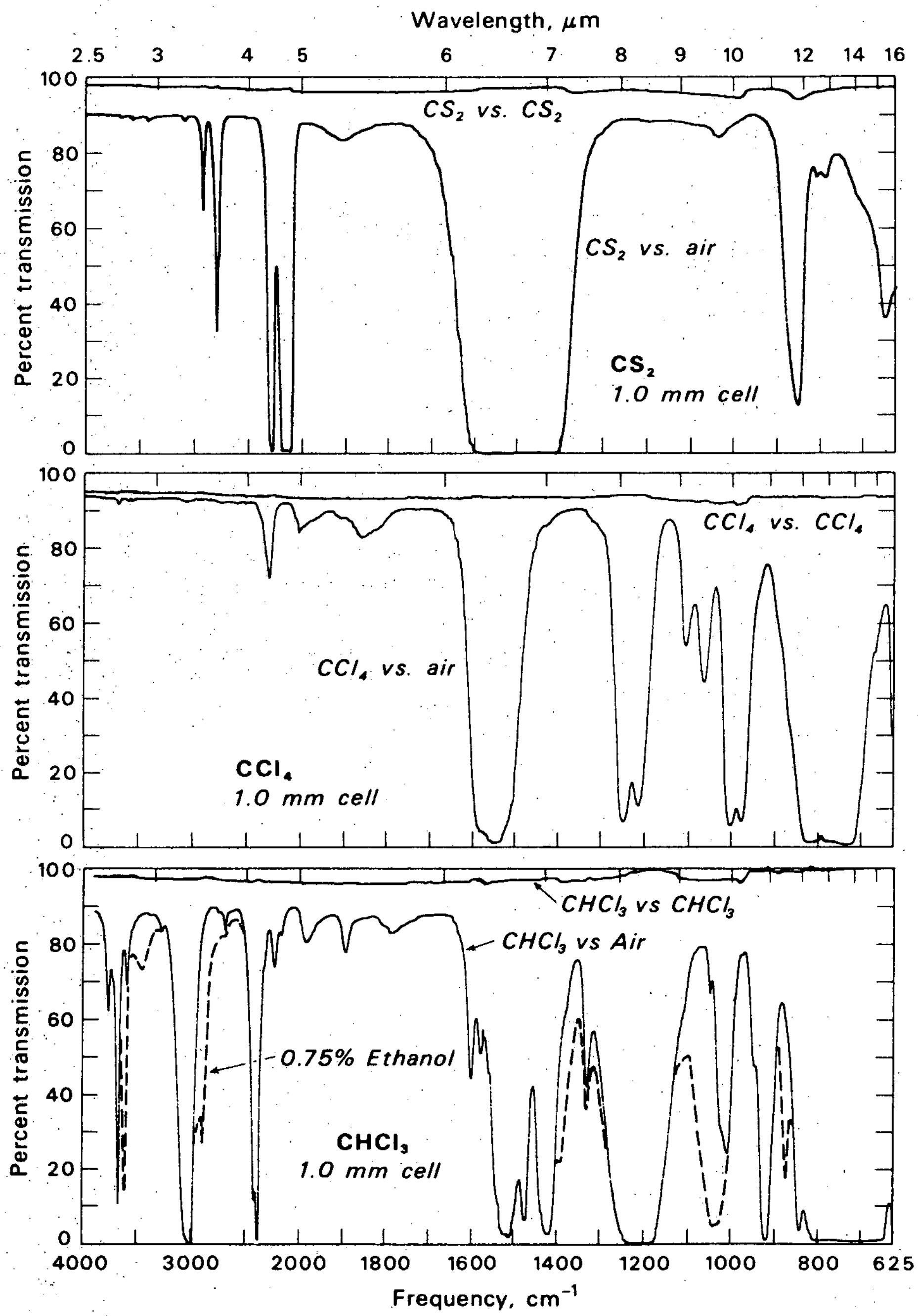
Fig. 20 <u>cis</u> double bond absorption in triglycerides. Solutions in CS_2, 40 mg/ml, 0.9 mm cells. Differential spectra, tripalmitin solution in reference cell. (From Ref. 68.)

Fig. 21 Infrared spectra (9.5 - 10.5 μm) of methyl oleate-methyl elaidate mixtures CS_2 solutions, 15 mg/ml, 3.5 mm microcell. (From Ref. 68.)

Fig. 22 Carbonyl absorption bands of representative serum lipid types. Concentration of each has been adjusted to a fatty acid content of 3.0 mg/ml. CCl_4 solutions, 1.0 mm cell. (From Ref. 14.)

Fig. 23 Infrared spectra of representative serum lipid types in the region of CH_2 bending absorption. Roughly comparable concentrations in CCl_4, 1.0 mm cell. (From Ref. 14.)

Fig. 24 Dotted curve is a computer plot of the recorded spectrum (1790-1690 cm^{-1}) of the mixture of three lipid components indicated. The approximately superimposed solid curve is the summation of individual pure component (lower) curves, calculated as a least-squares fit. (From Ref. 14.)



DBL 701-5508

Fig. 1

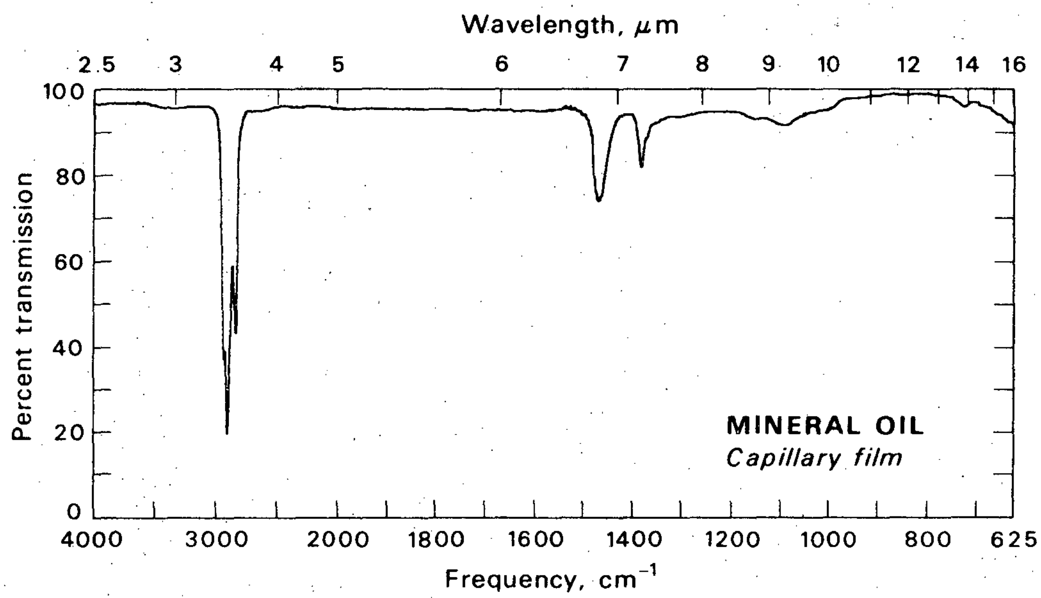


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Fig. 2

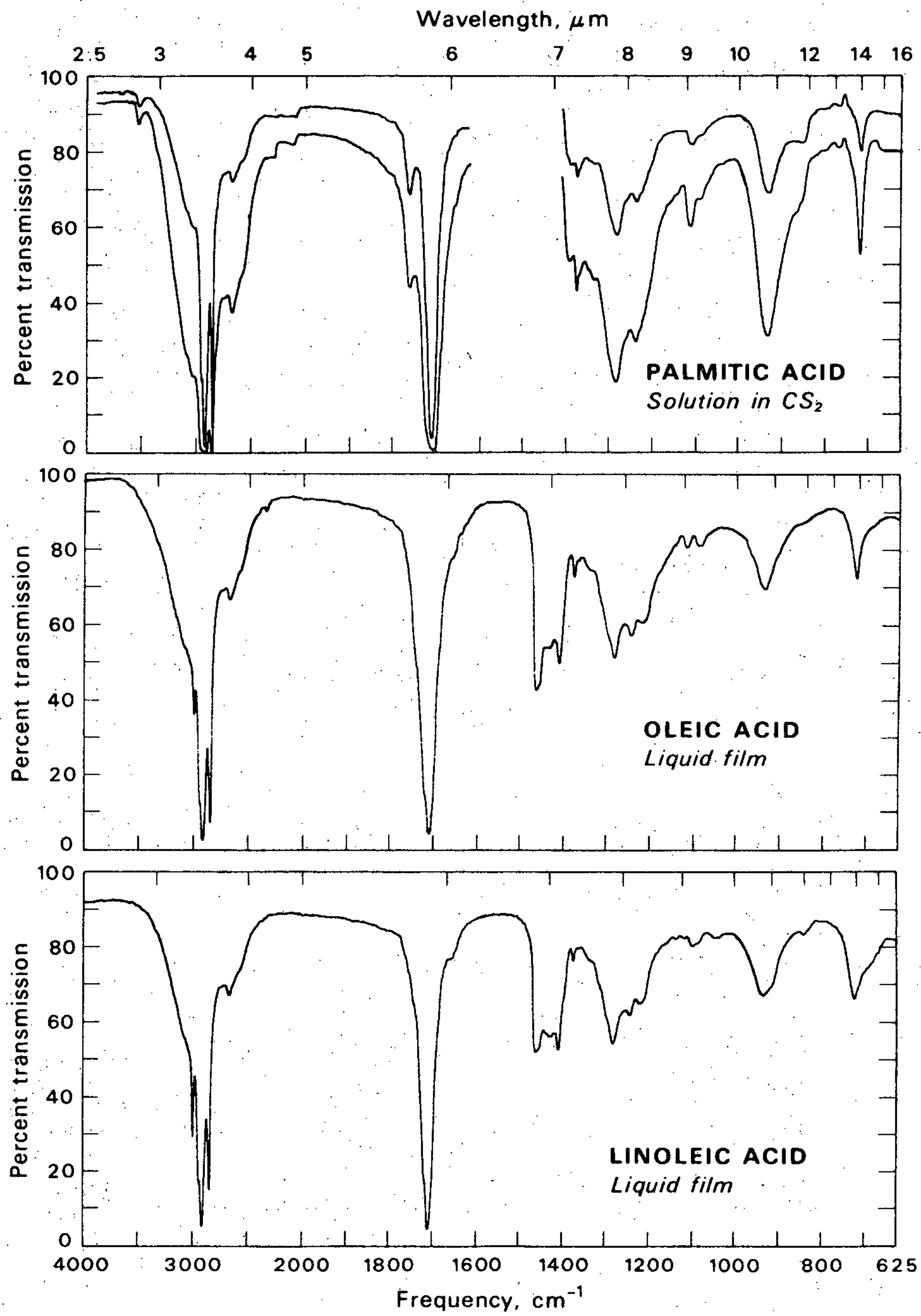

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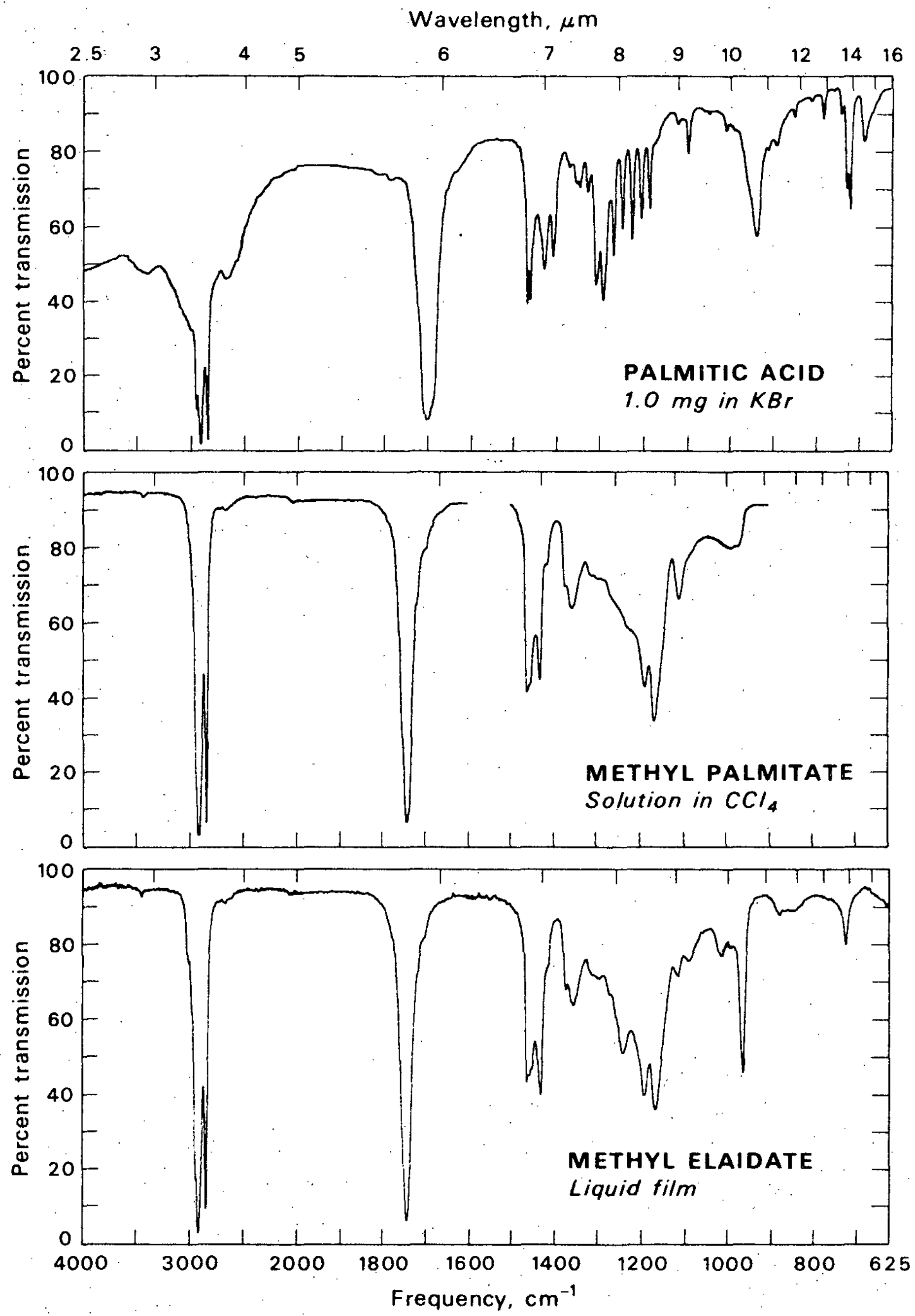
Fig. 3



DBL 701-5509

Fig. 4

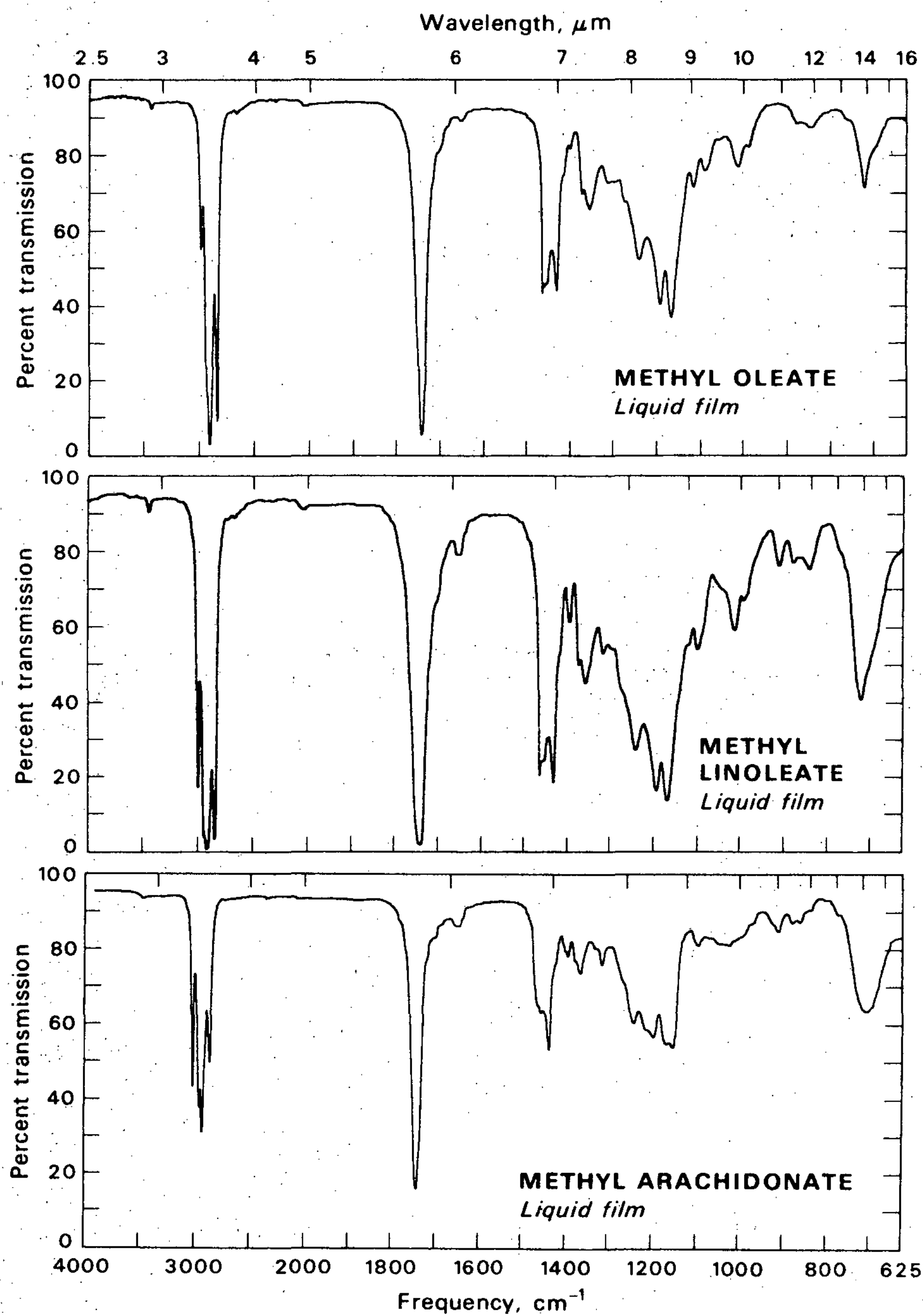


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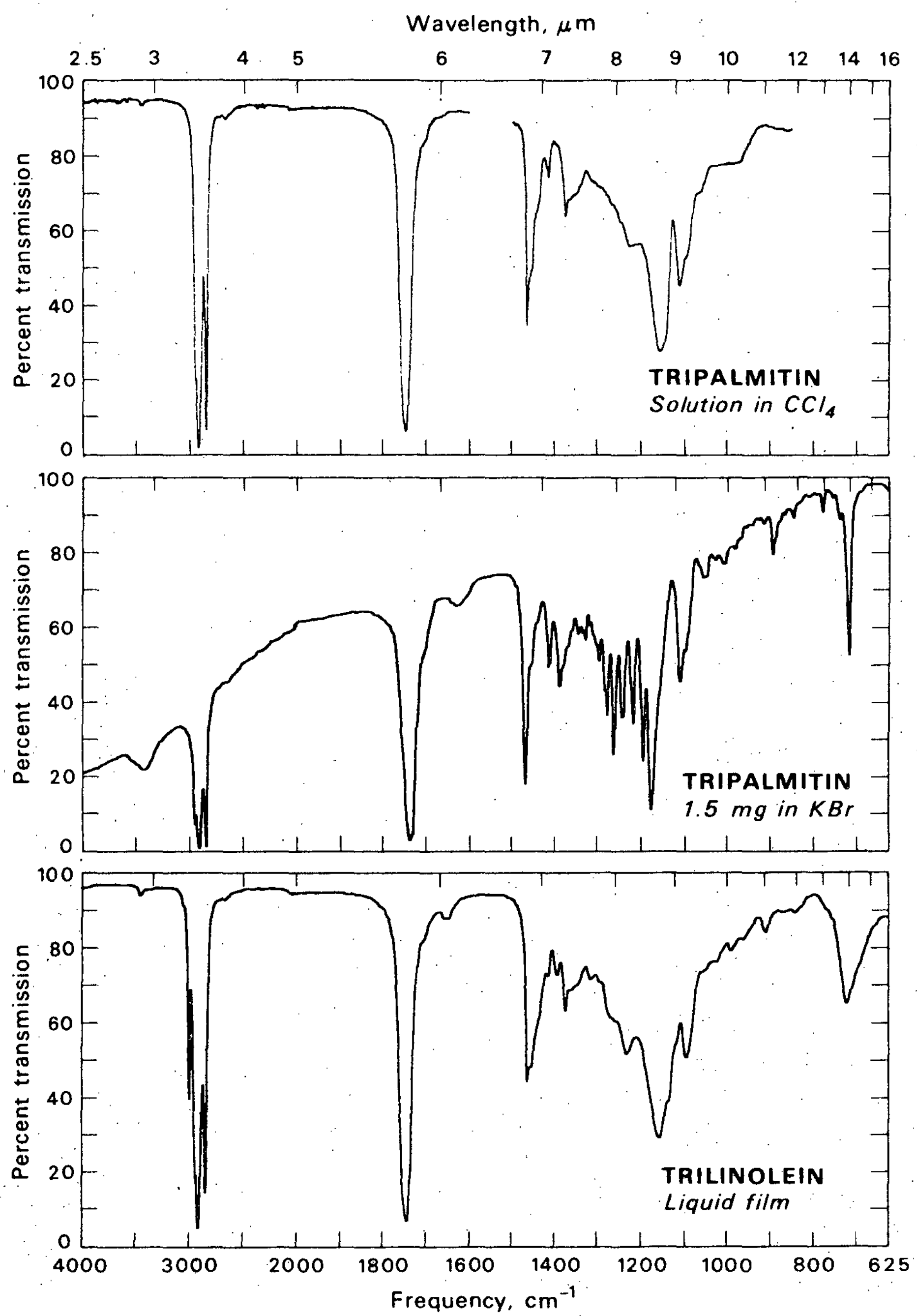
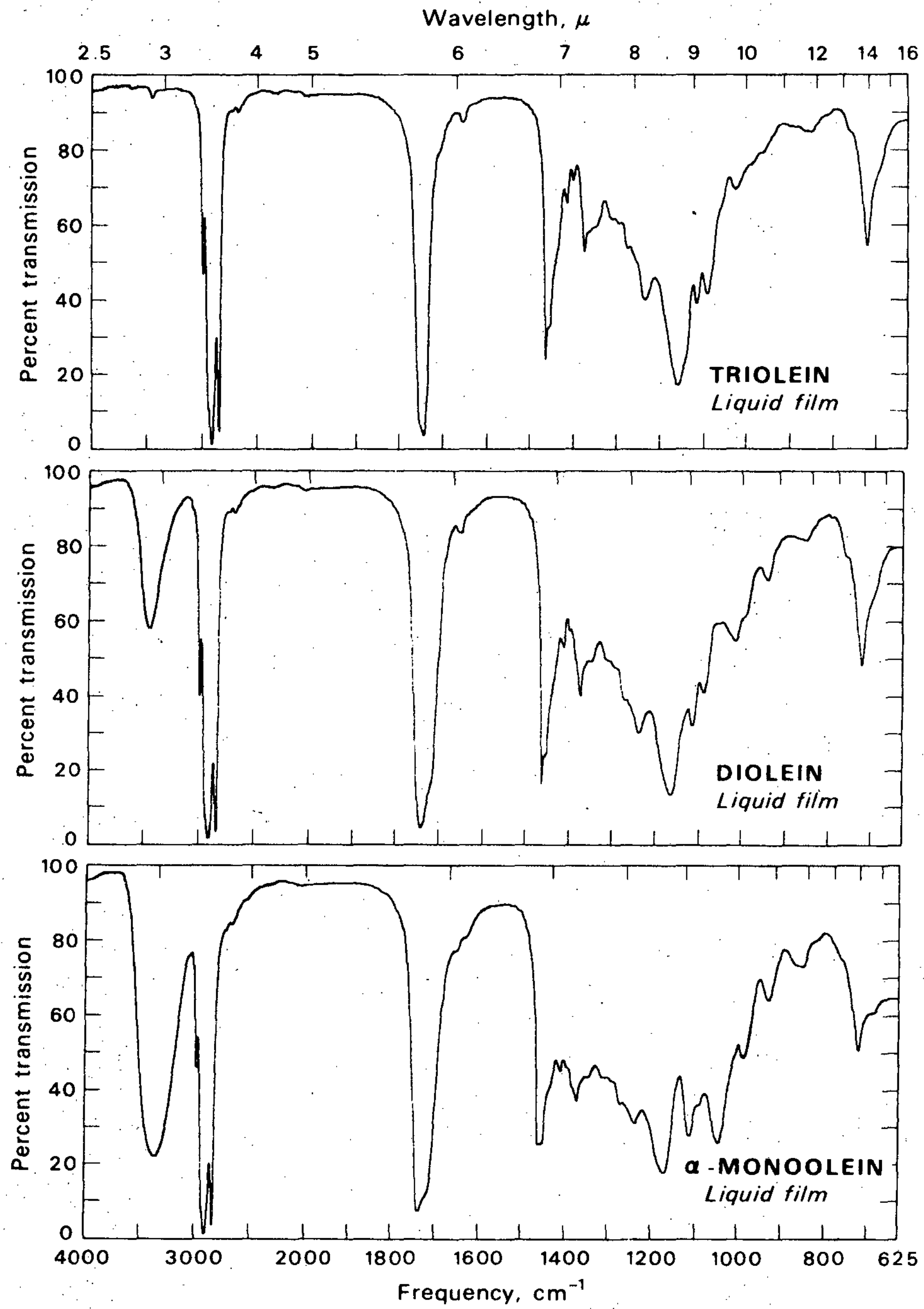
Fig. 5



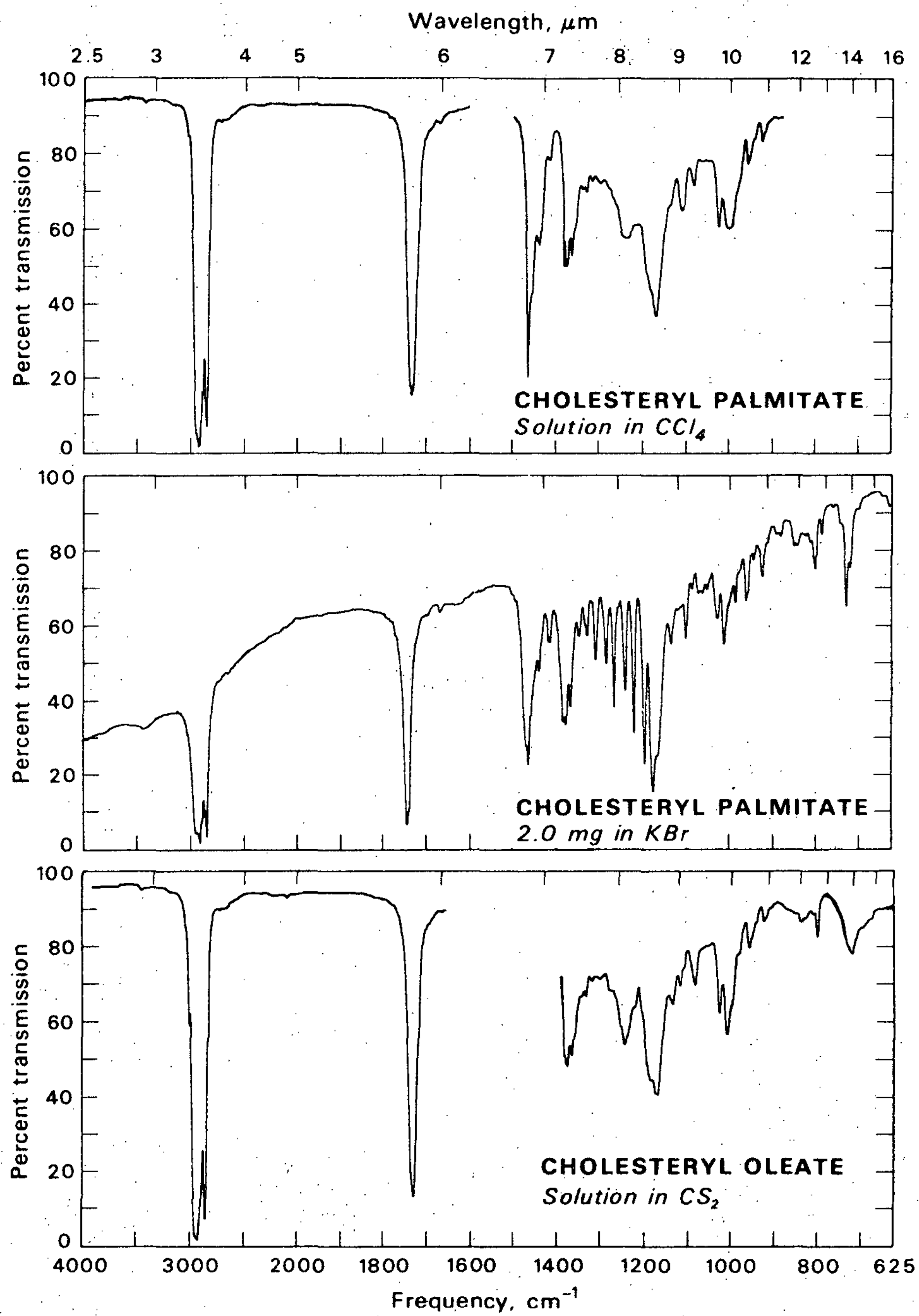
DBL 701-5511

Fig. 6



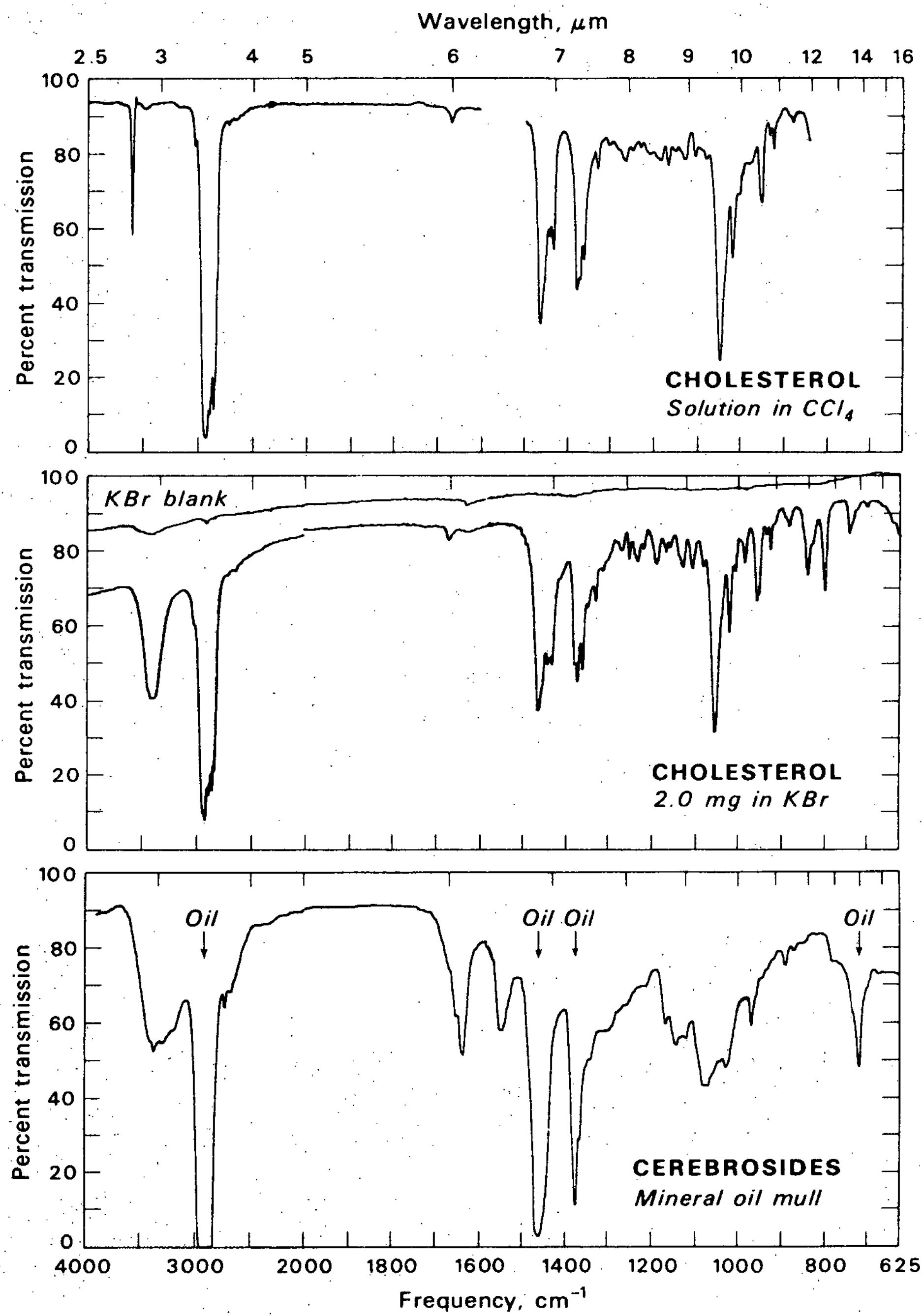
DBL 701-5512

Fig. 7



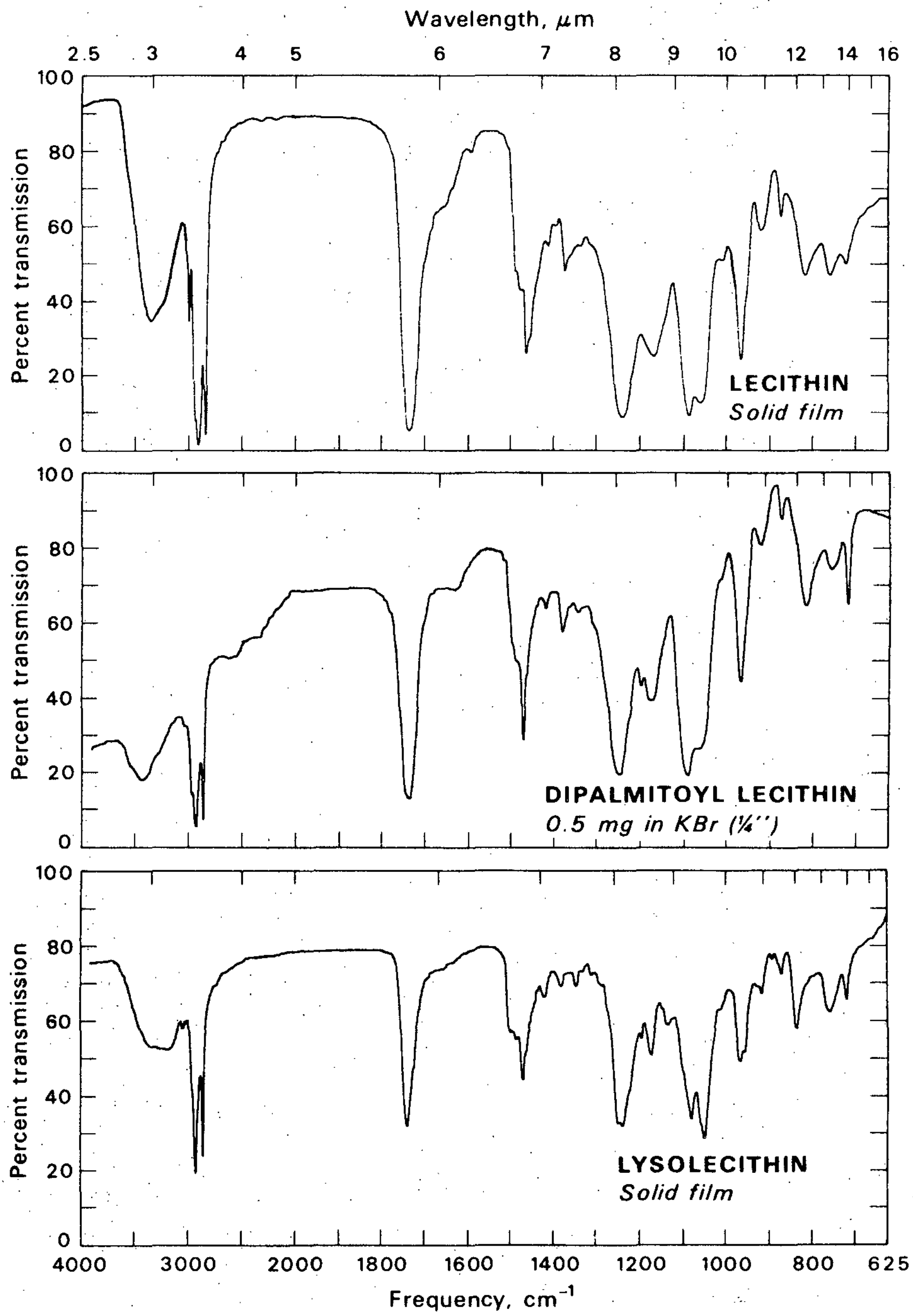
DBL 701-5513

Fig. 8



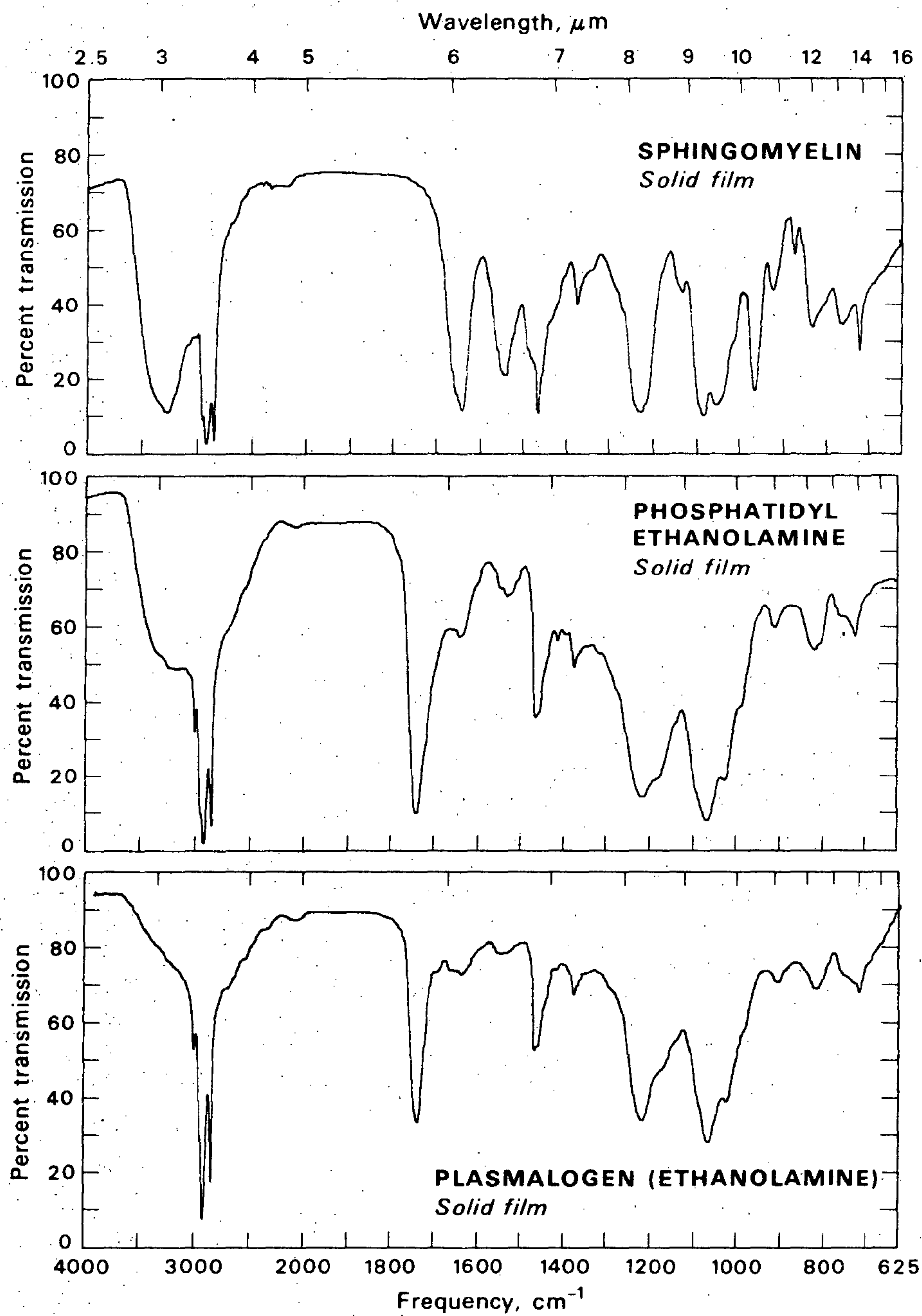
DBL 701-5514

Fig. 9



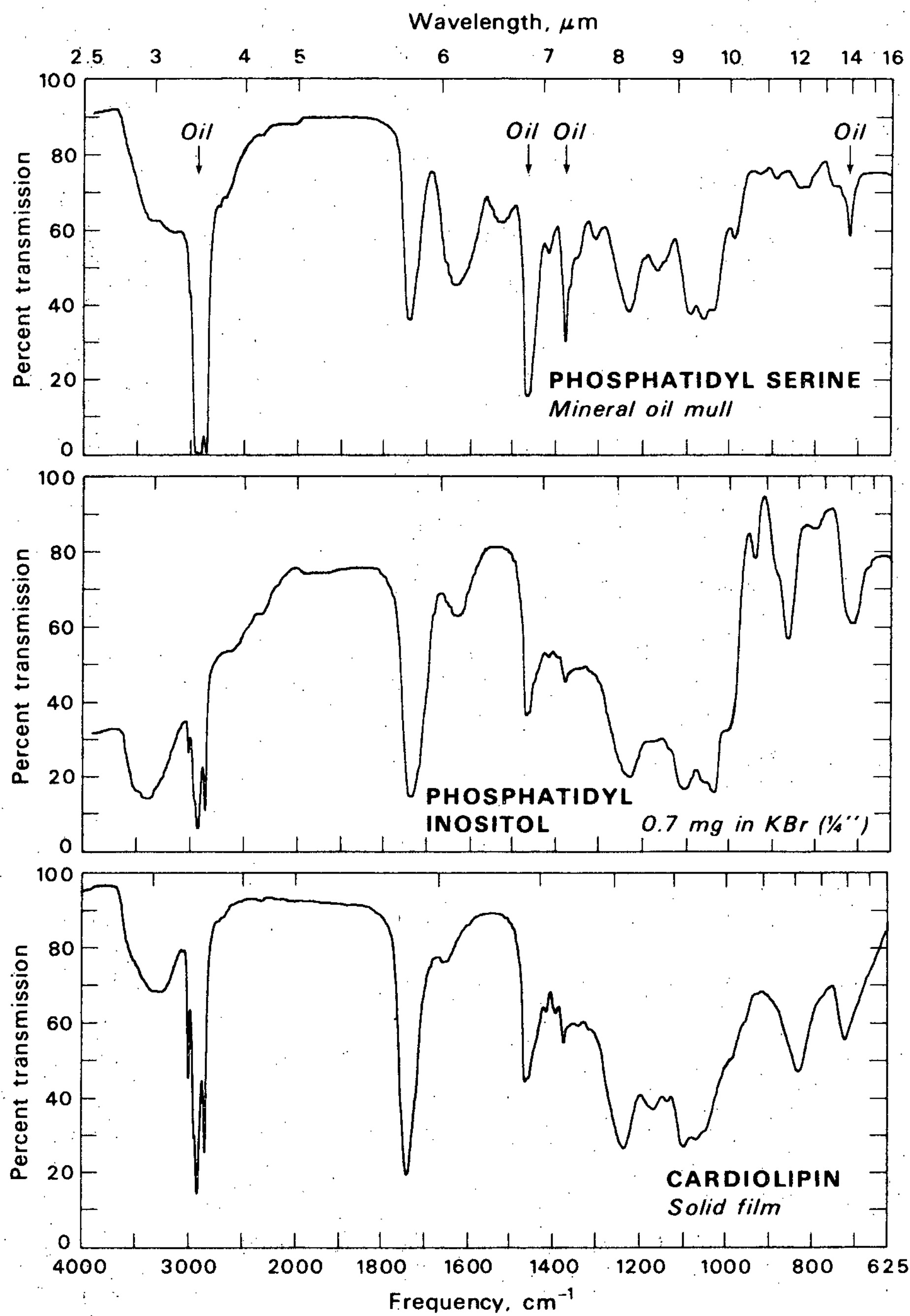
DBL 701-5515

Fig. 10



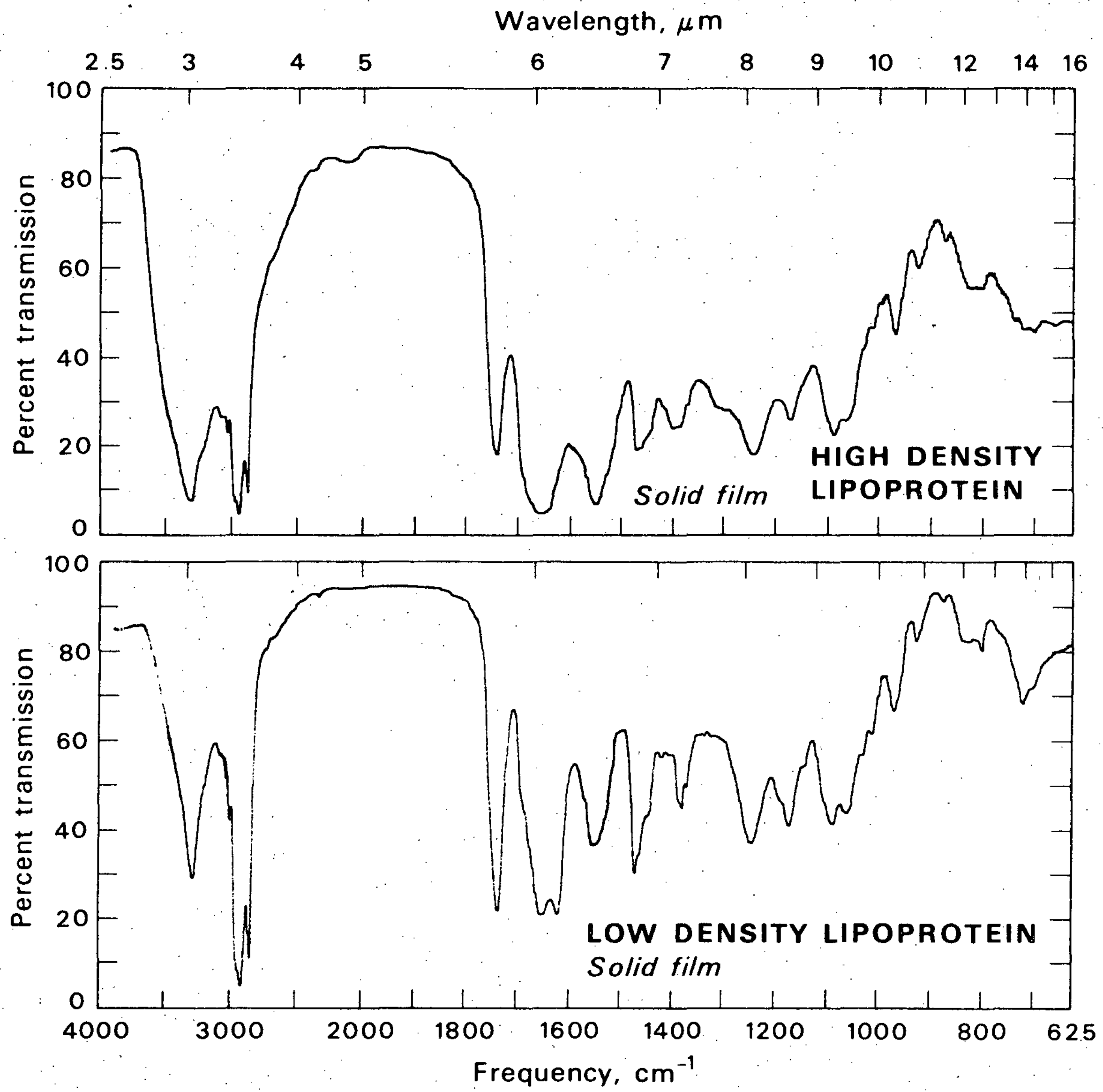
DBL 701-5506

Fig. 11



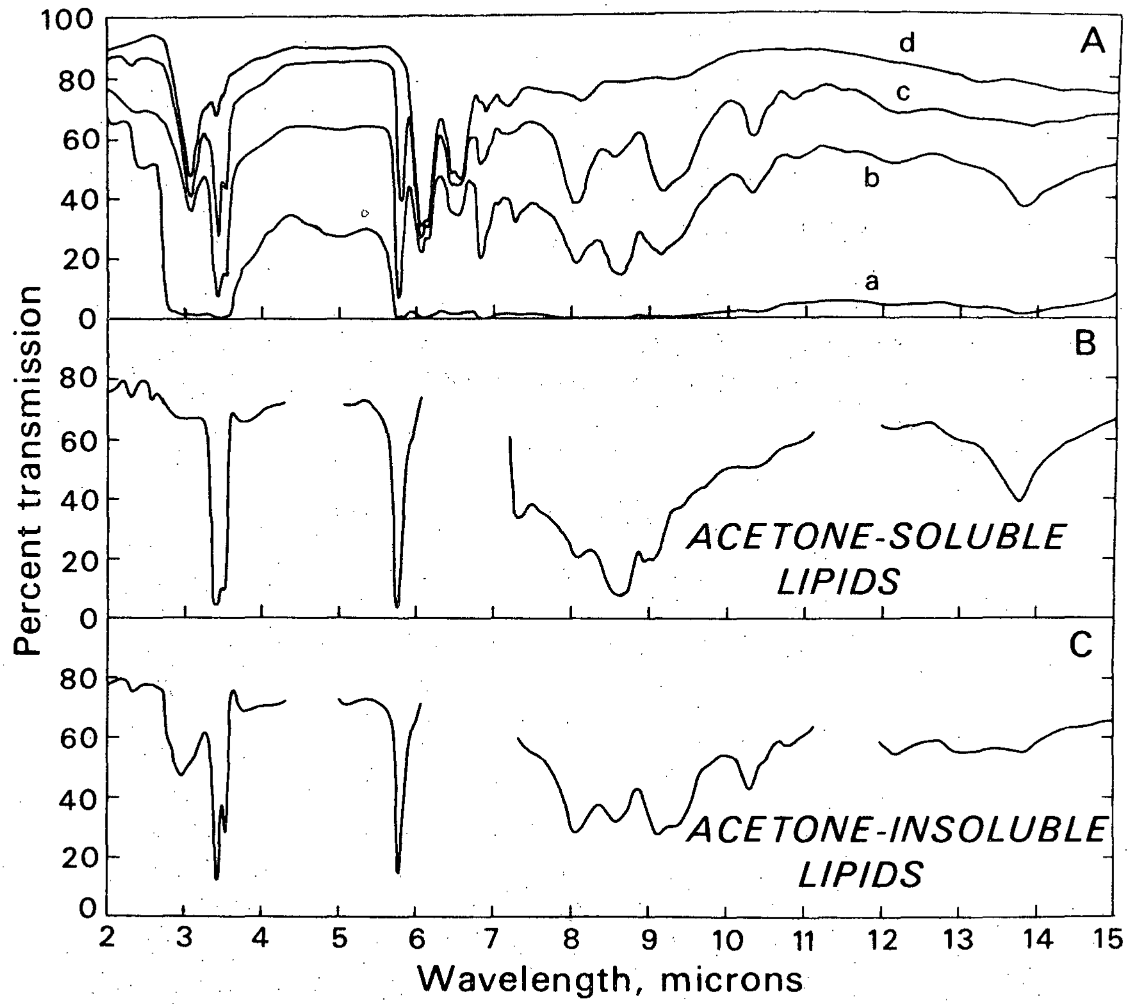
DBL 701-5516

Fig. 12



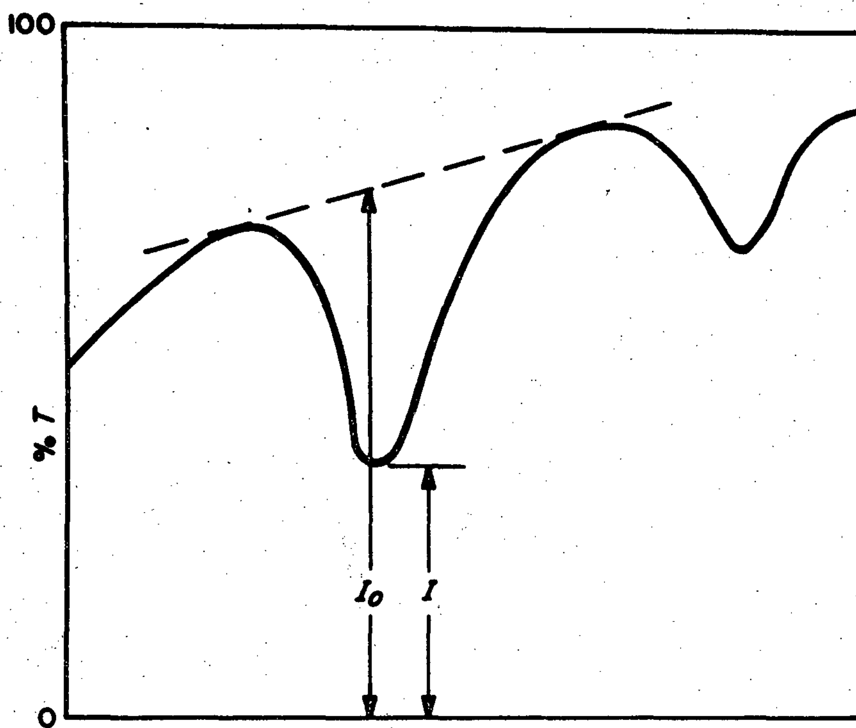
DBL 701-5507

Fig. 13



DBL 701-5504

Fig. 14



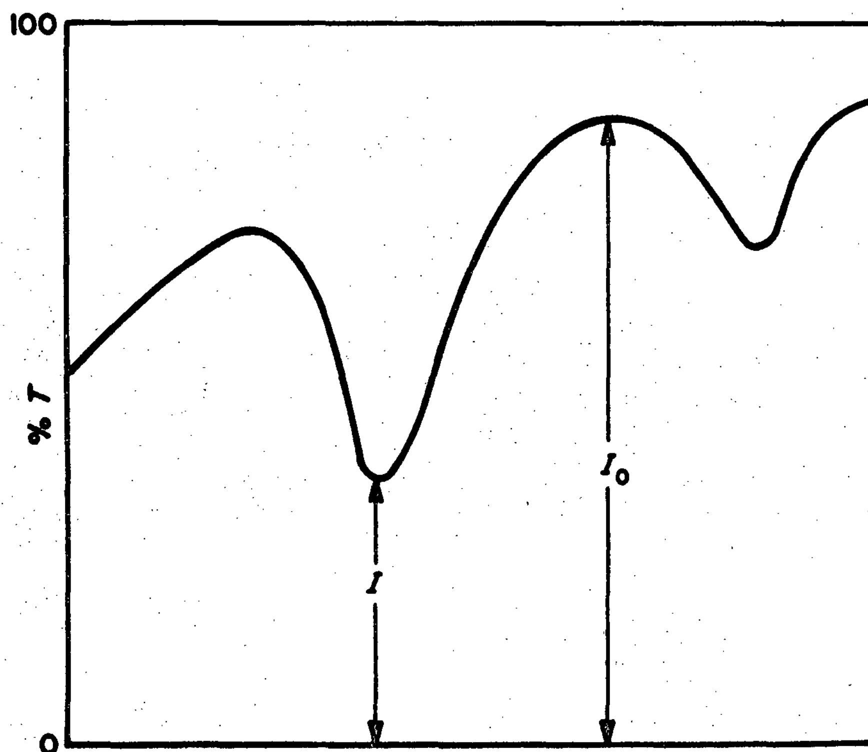


Fig. 15

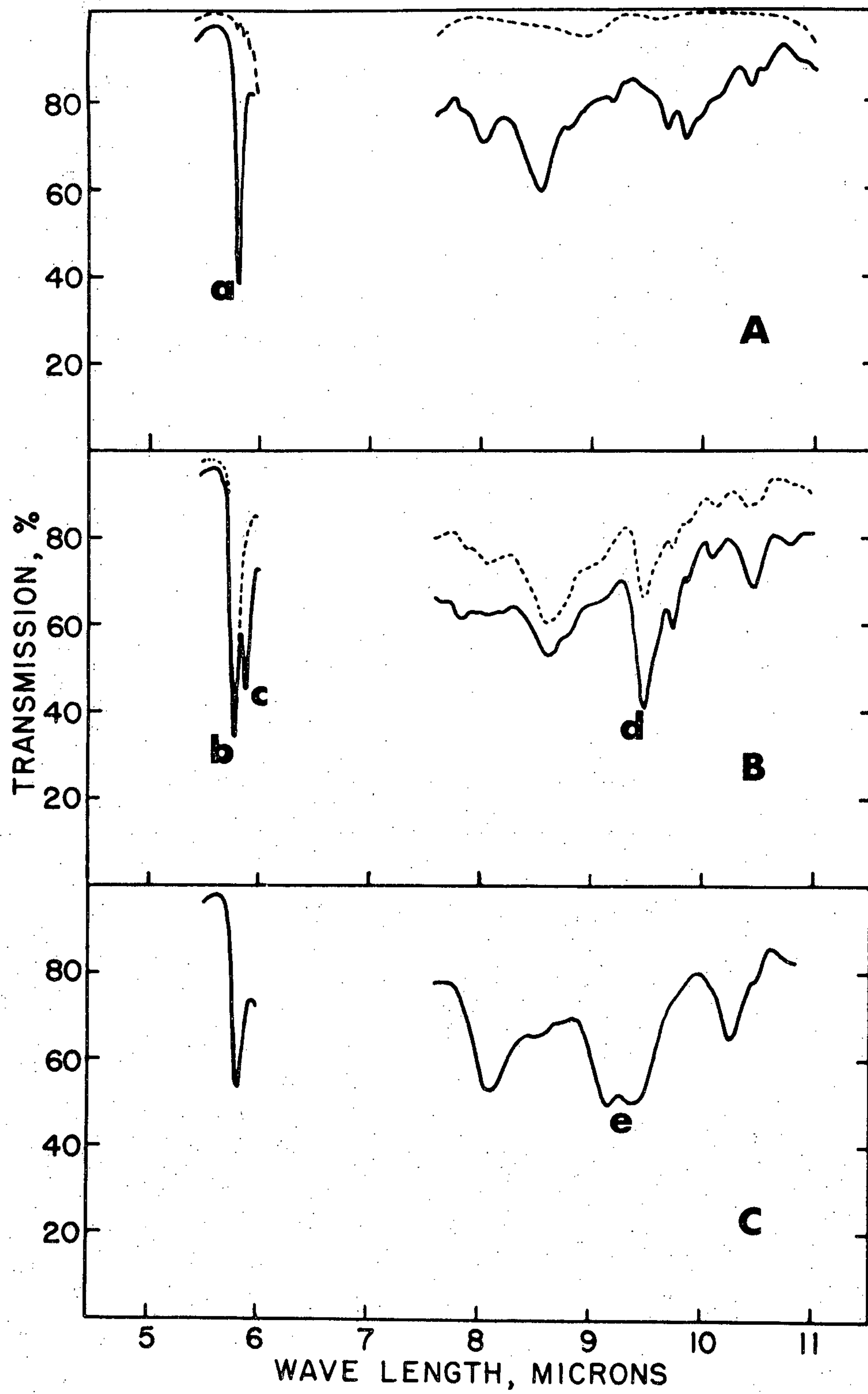


Fig. 16

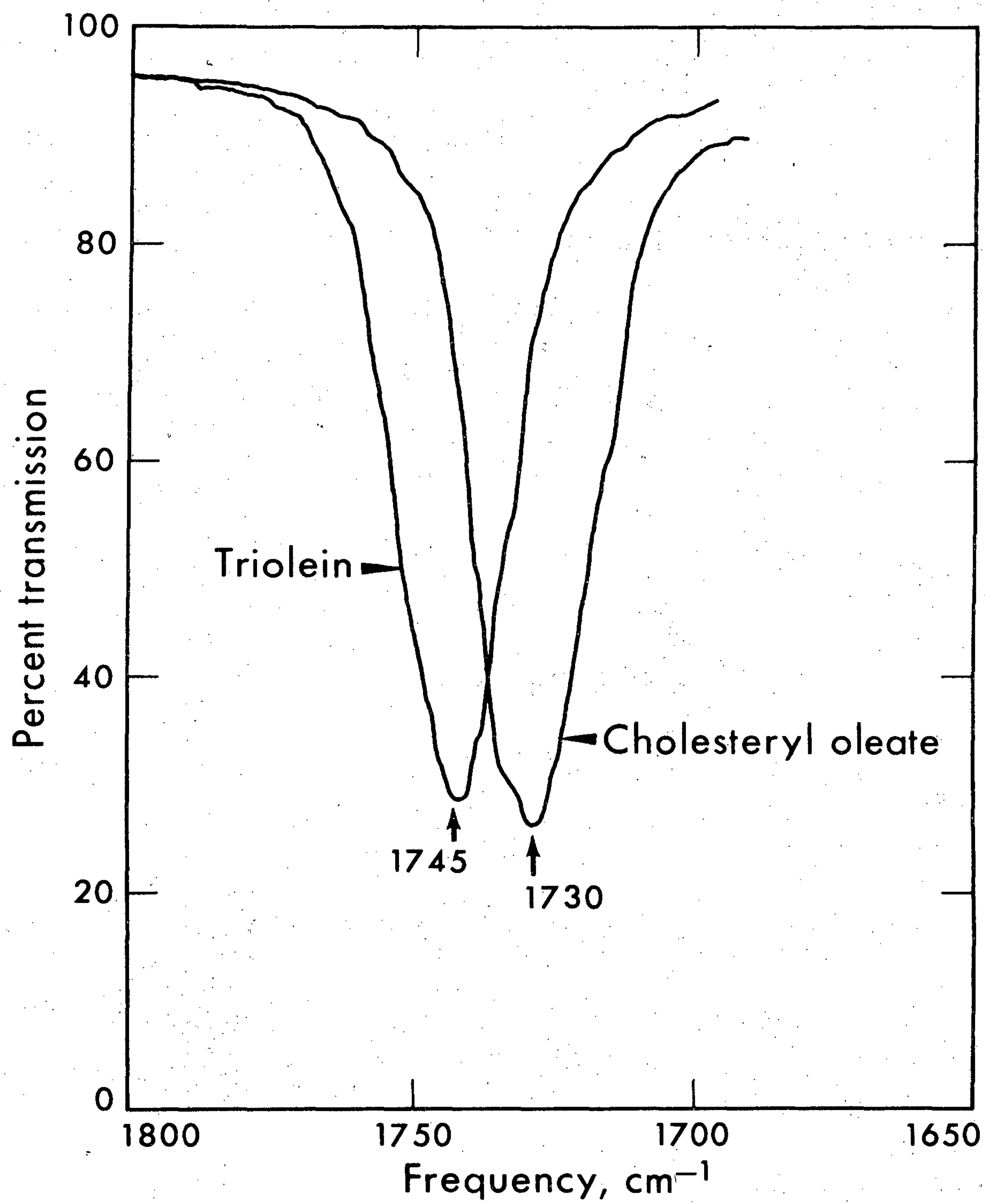
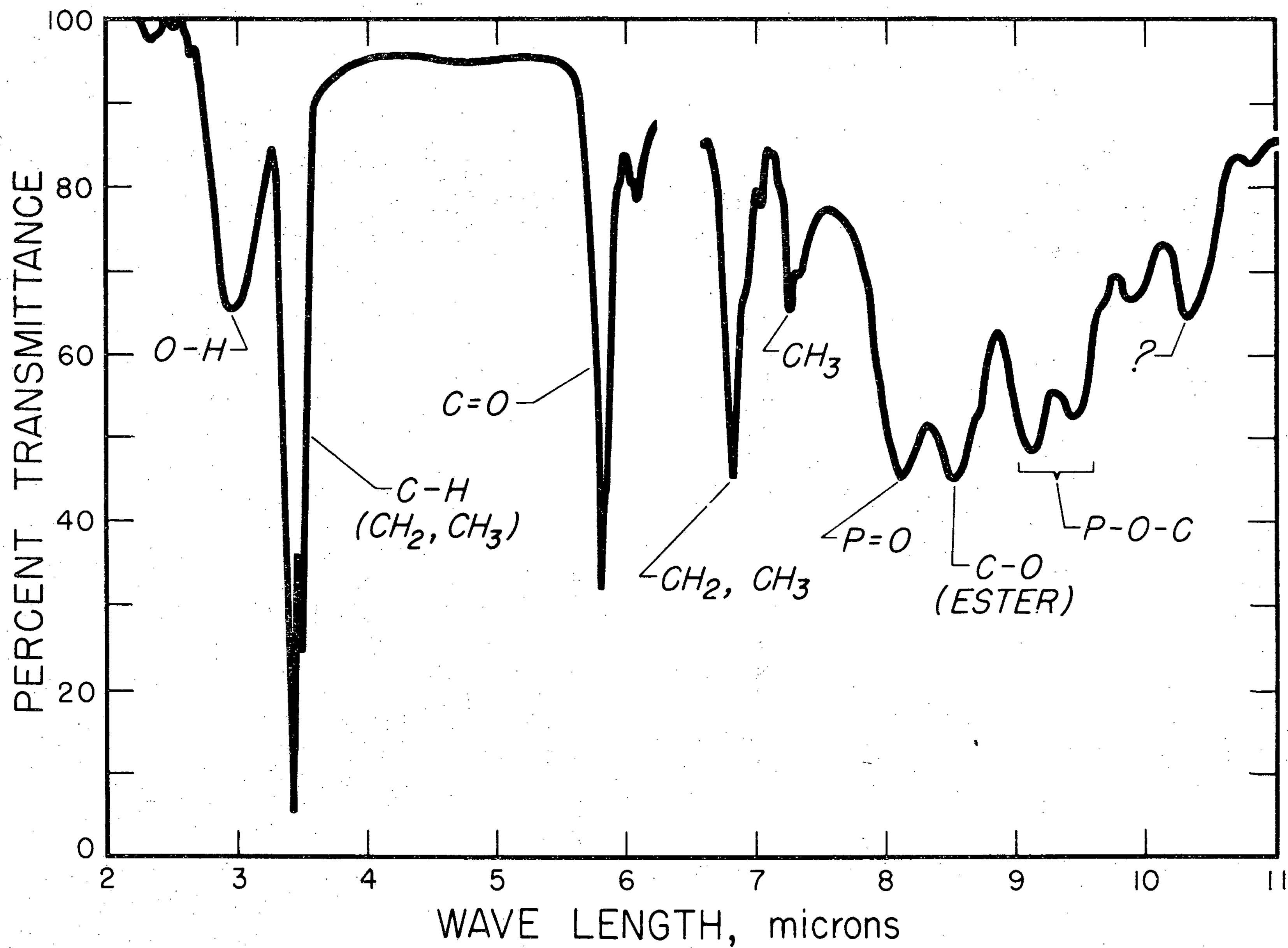
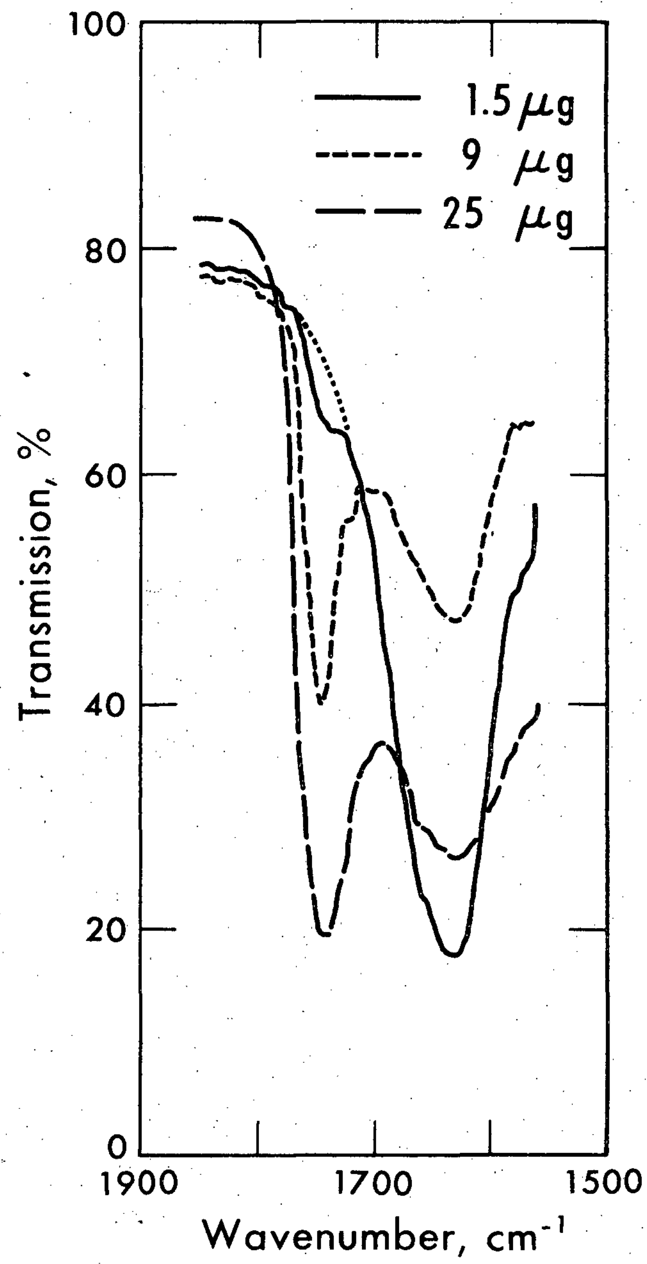


Fig. 17



MU-12507

Fig. 18



MUB-12755

Fig. 19

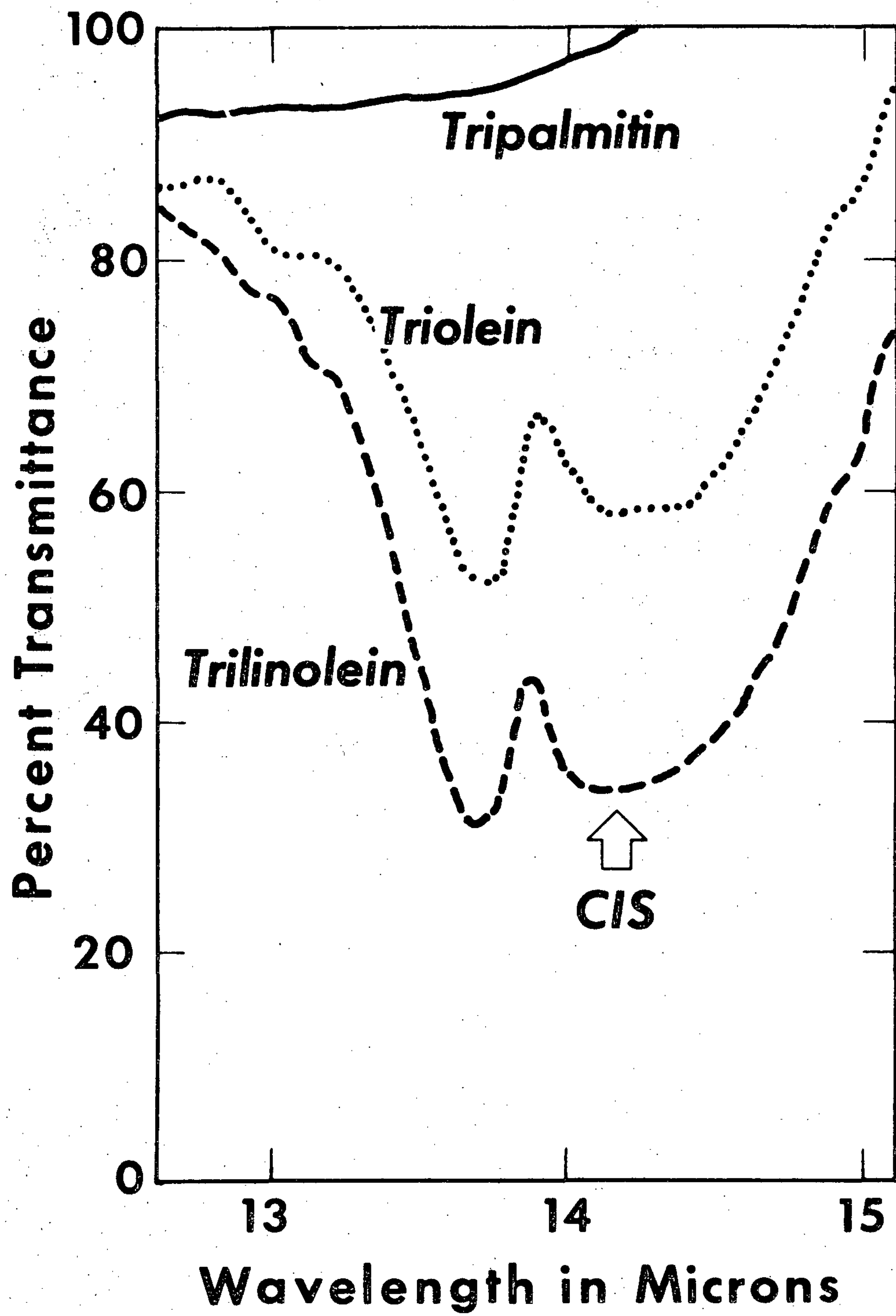
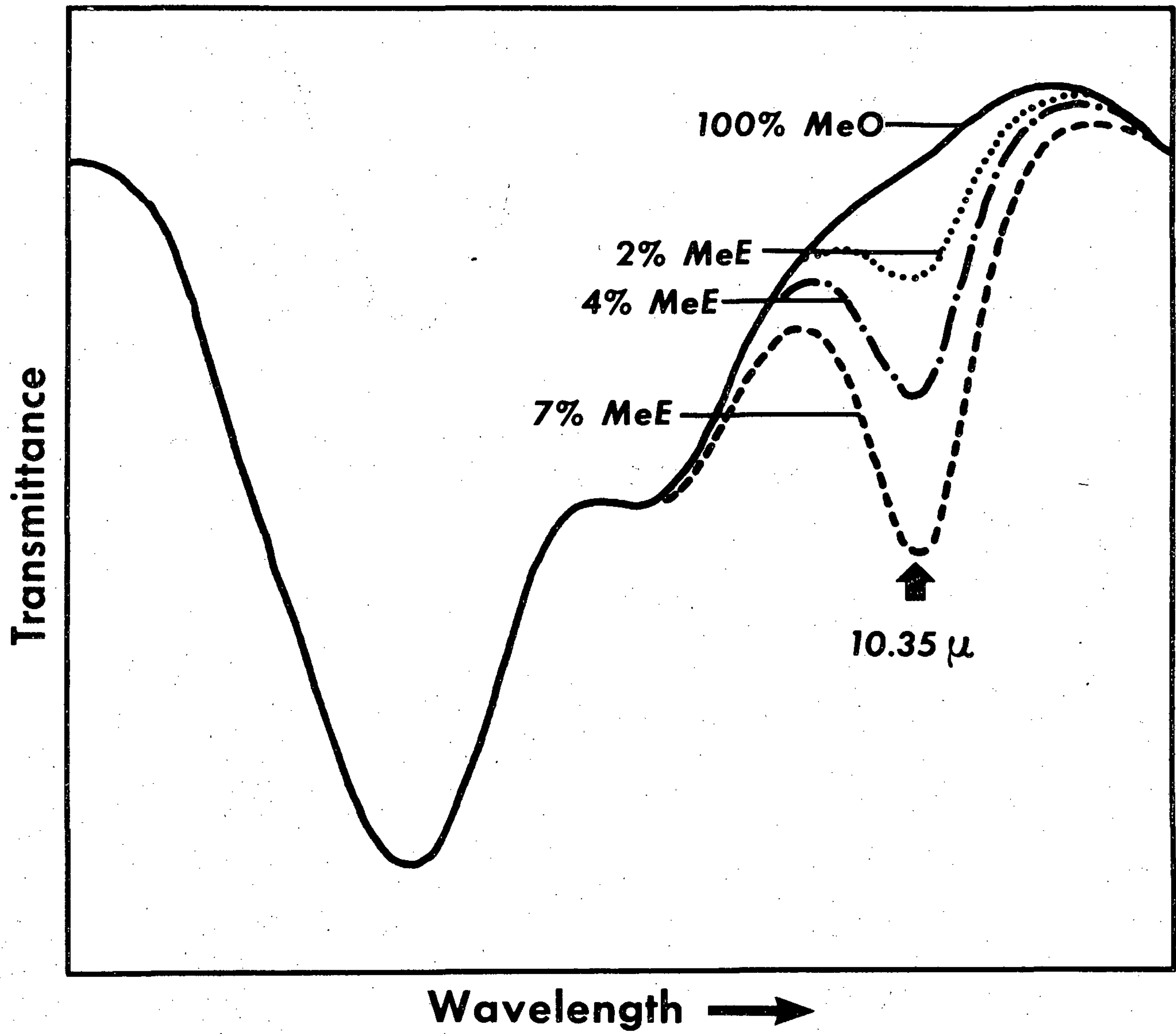
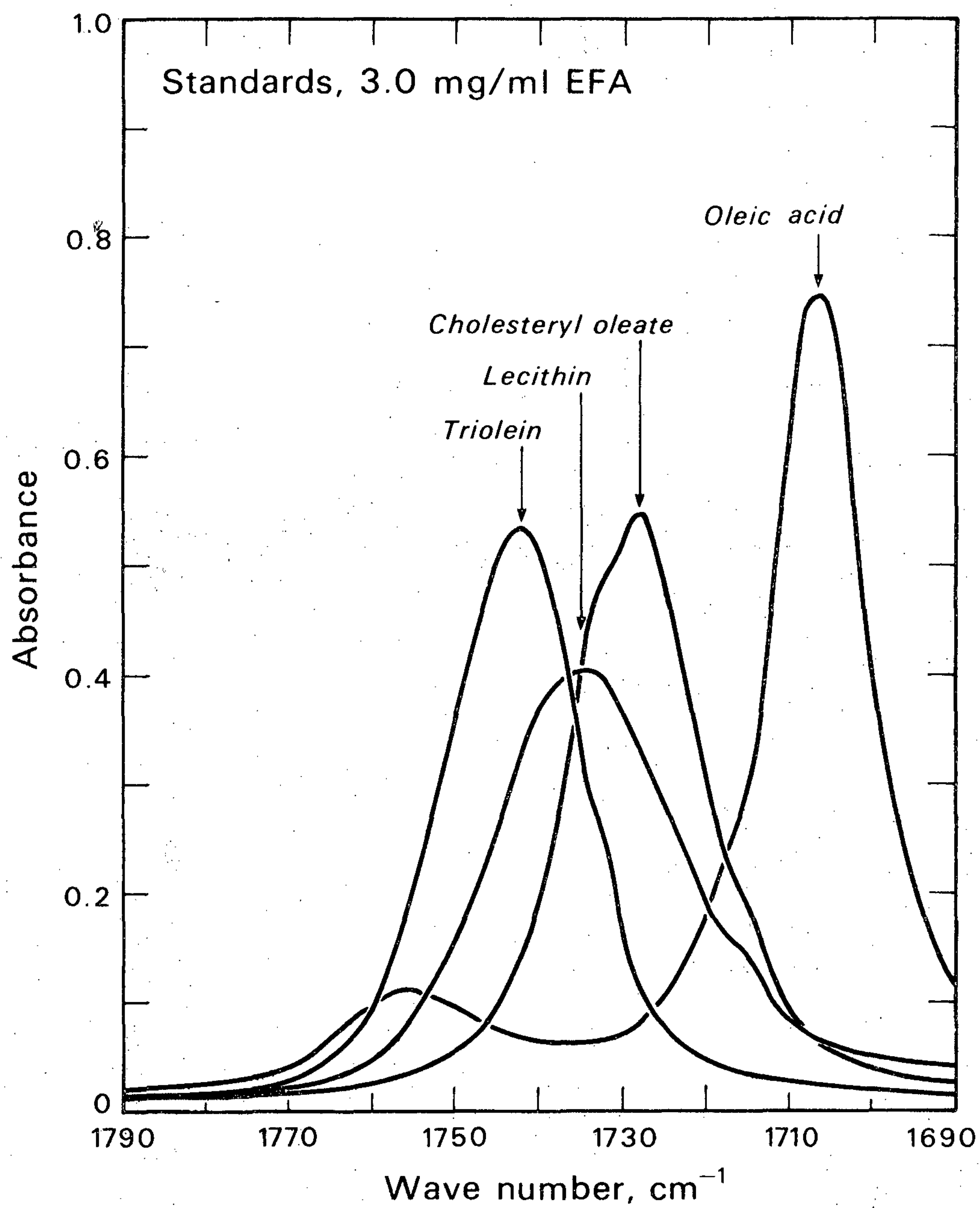


Fig. 20



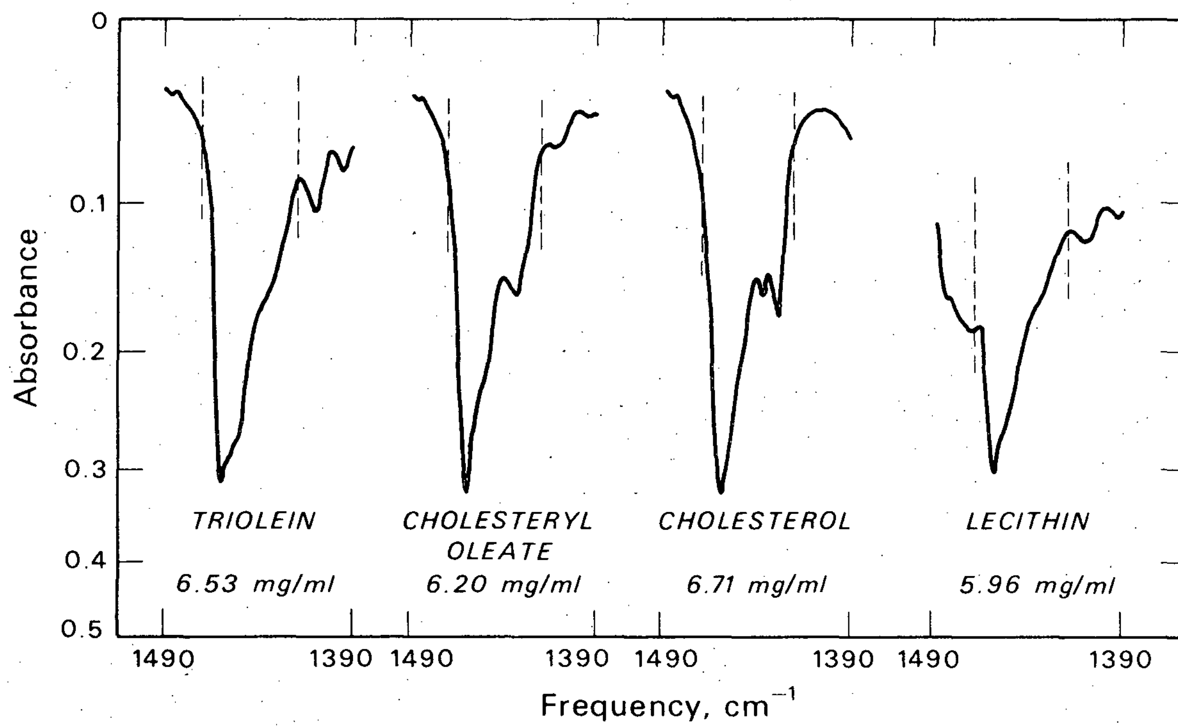
MU-24566

Fig. 21



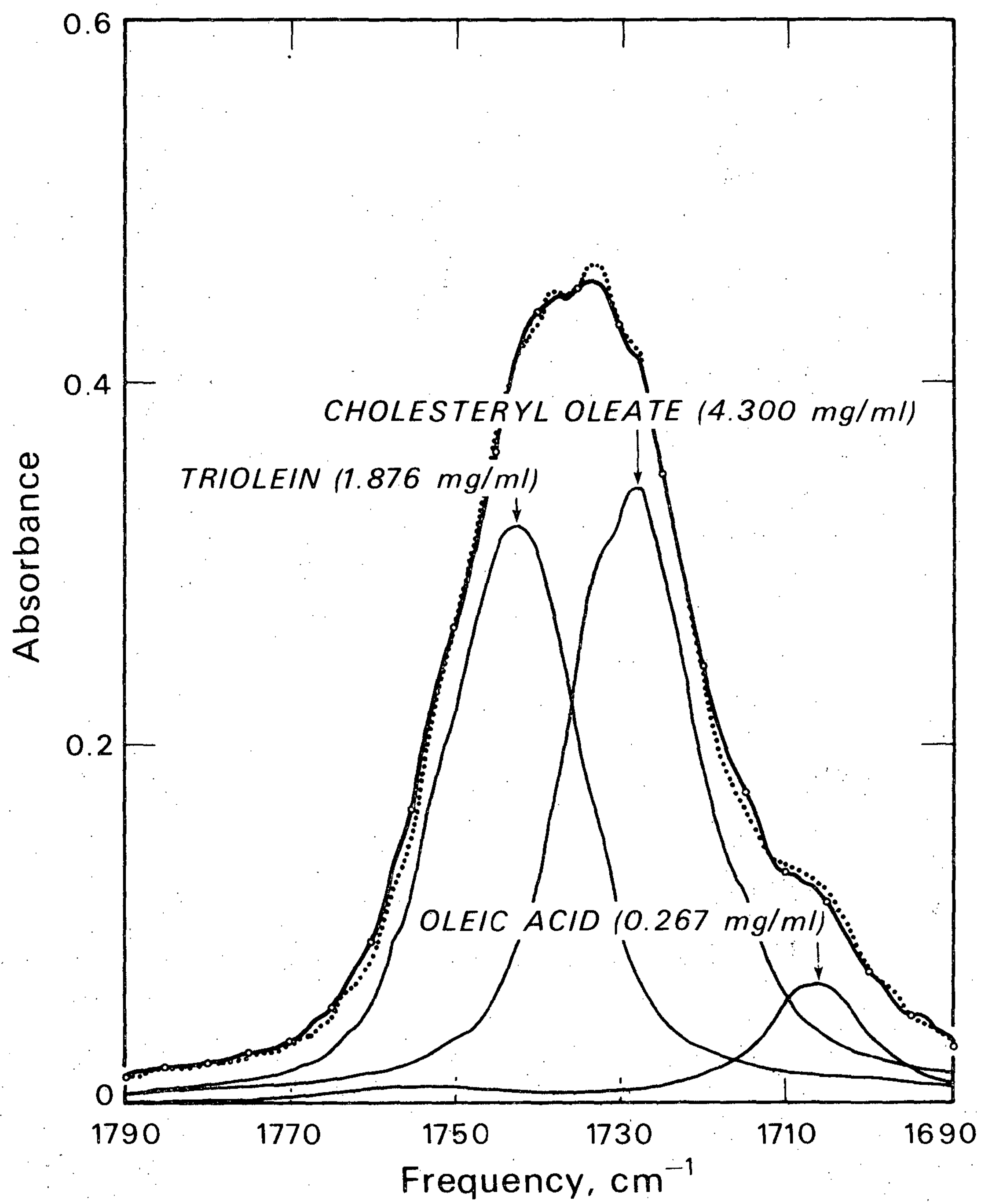
DBL 682-4589

Fig. 22



DBL 683-4636

Fig. 23



DBL 683-4634

Fig. 24

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