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Infrared Spectroscopy of Lipids

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Abstract

During the last ten years, considerable advances have been made in the application of infrared spectroscopy to the study of lipids. This work reviews this contribution.

Starting with a brief discussion of the basis of the technique, applications of a qualitative and quantitative kind are described. The lipids discussed include the simple fatty acids, alcohol esters, then the more complicated glycerides, cholesterol esters, soaps, phospholipids, lipoproteins, and finally some recent studies of the myelin sheath.

IN THE LAST TEN YEARS increasing use has been made of infrared (IR) spectroscopy for qualitative and quantitative estimations on lipid material. (1–3) Spectroscopic studies of solid state behaviour combined with X-ray methods have also been increasing in number and are helping to reveal structural information about polymorphic transitions and about the liquid crystalline transitions which occur with certain lipids. Lipids have now been examined in the vapour, liquid and solid state as well as in solution. Because of the long hydrocarbon chains present in most lipid material different crystal forms occur and this can bring complications to the IR spectra of the material in the solid state. At the same time these spectra can provide a great deal of useful additional information.

Theoretical Considerations

IR spectra arise from the interaction between matter and electromagnetic radiation of wavelength between about 1 μ to 50 μ (10,000 to 200 cm^{-1}) [most commercial instruments cover the region from 2 to 15 μ (5,000 to 650 cm^{-1}) but some recent instruments extend the range from 0.6 μ to 25 μ ($\sim$15,000 to 400 cm^{-1})]. The atoms of a molecule can vibrate in a number of modes, and for absorption of energy to occur it is essential that there should be a change in the dipole moment of a molecule during the vibration. The number of normal modes of virbation for a nonlinear molecule is equal to 3n–6 (where n = number of atoms in the molecule). Each normal mode of vibration can occur independently of the other modes, and the absorption band for each mode is known as its fundamental band whose position is defined by its fundamental frequency. The fundamental frequencies are usually found in the 2–15 μ (5,000 to 650 cm^{-1}) region. Most of the vibrational modes involve the very complicated motion of all the atoms, and the particular values of the frequencies are, therefore, highly characteristic for a particular molecule. In some cases the mode of vibration is highly localised in the molecule and involves the motions of atoms in a particular group giving rise to a characteristic group frequency. Empirically it has been shown that these frequencies are almost independent of the structure of the remainder of the molecule and hence afford a good diagnostic method for ascertaining the presence

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of specific groupings. The environment of the group can modify the frequency slightly. It is thus possible to deduce from the wavelength of the carbonyl absorption band whether (for example) the environment of this grouping is a carboxylic acid or ester. Similar considerations apply to other groupings. Correlation charts are valuable for summarising the empirical information relating band frequency to the particular groups present. Reference spectra are also available.

An unknown substance can usually be identified by comparison with the spectra of molecules of known constitution. When a perfect match between spectra is obtained, identification is complete. This procedure can be carried out quite rapidly with IR absorption data arranged on punched cards by sorting through the cards for the particular card containing the main absorption bands corresponding to those of the unknown material. Even if complete identification is not possible by this means, usually two or three compounds giving somewhat similar spectra can be found, from which some information can be deduced about the structure.

When the substances are examined in the solution state the bands rising from the particular functional groups can usually be readily identified. However, when the materials are examined in the solid state the spectra are considerably more complex and many additional bands are observed. We shall discuss this in more detail later. In addition to this, occurrence of polymorphism complicates the situation considerably. As many as five or six polymorphic forms may be obtained for any one compound and as many as five or six different IR spectra may be obtained. This means that for identification of known and unknown material, both have to have exactly the same pretreatment before the spectra are obtained, e.g., crystallisation from the same solvent at the same rate and at the same temp. Orientation and partial polarisation of the radiation within the spectrometer can cause intensity variations which require caution. Polarisation data may also be obtainable on solid state materials and can give information about the orientation and packing of the lipid molecules in the solid state.

The IR spectra of compounds containing asymmetric carbon atoms are interesting. In solution and in the liquid state the spectra of enantiomorphs are identical. However, in the crystalline state the spectrum of the racemic compounds can differ from that of the D or L rotatory isomers because of differences in the manner of packing in the crystal. (The spectra of diastereo-isomers are different in all physical states). The polymorphism of the isomers may also differ from that of the racemic material.

Instruments and Techniques

Considerable progress has taken place in recent years with the production of commercial IR spectrometers. Nowadays most instruments are double-beam in operation.

In a typical modern IR spectrometer the source of radiation is a water-cooled Nernst filament, and the dispersion unit a rock salt prism Littrow monochromator with an off-axis paraboloid collimating mirror. The detector is probably a Golay pneumatic cell having a large receiving area and good mechanical robustness. The output of the detector is modified by a simple and stable electronic amplifier and fed to a homodyne rectifier which gives a d-c output proportional to the difference in intensity between sample and reference beam. The difference signal is fed to a servo mechanism which adjusts a beam attenuator in the reference beam until the difference signal is reduced to zero. The percentage transmission is recorded linearly in wavenumbers (cm^{-1}) or in wave length (μ) by means of marker pens. The optical system in some cases can be evacuated to prevent the effects of strong atmospheric absorption. Gratings are usually provided as accessories, thereby producing prism-grating double monochromator instruments of high resolving power and low stray light. The radiation enters the grating monochromator through a curved entrance slit; it is dispersed and then enters the prism monochromator through a wide intermediate slit. Radiation from the prism leaves through a straight exit slit and is condensed on to the thermal detector. The curvature of the entrance slit compensates for image curvature introduced by the prism monochromator.

Cell windows are usually made of rock salt or potassium bromide. For gaseous samples, fairly long cells 1 to 20 cm may be used, and with a fixed length cell the pressure of the gaseous sample may be varied to achieve a suitable optical density in the wavelength region of interest. Liquids may be examined for qualitative analysis by using rock salt cells which vary in thickness from about 0.01 mm to several millimetres, or quite frequently by squeezing between rock salt plates.

Solid and liquid samples may be examined in solution and this is most frequently used for quantitative analysis. Suitable solvents are a problem because of their absorption in the IR region. Water is not often used because of its high absorption and because most work is carried out with cells made with rock salt windows. Carbon tetrachloride and carbon disulfide are used because they have considerable transparent regions. Other solvents used include acetone, methyl formate and nitromethane. A variable path cell is of considerable convenience for balancing out the solvent absorption when using a double-beam spectrometer. Solid samples may be examined after melting between rock salt plates or by making the sample in a medium such as Nujol or perfluorokerosine, or by pressing the sample into a plate with potassium bromide or potassium chloride. The use of low temp and high temp cells is also particularly valuable with lipid materials either to study their polymorphic behaviour or to confirm the identification of a compound by obtaining more than one spectrum. We shall discuss many applications of this. Beam condensing units are commercially available and are useful for examining small samples of lipid material.

A method for obtaining the IR spectra of small samples obtained by thin-layer chromatography (TLC) has been described. (4) A circle of Kraft paper the same size as a normal potassium bromide disc but having a small hole, 2.4×7 mm at its centre, is placed in the press. The amt of sample is reduced to 50–100 μg and mixed with 20–30 mg of potassium bromide. The charge is placed in the hole and the disc pressed in the normal manner. The finished product consists of a potassium bromide window mounted in a paper circle. A corresponding mask for the reference beam is required.

Quantitative Measurements

The basis of quantitative measurements with IR spectroscopy is similar to that of ultraviolet or visible spectroscopy.

Intensity

To describe the intensity of a band the Beer-Lambert relationship is used.

$$\log_{10} \frac{I_o}{I} = E = Kcl,$$

where I_o is the intensity of the incident radiation
I is the intensity of the transmitted radiation
c is the concn in gram/litre
K is the specific extinction coefficient
l is the path length in centimetres

$\log_{10} \frac{I_o}{I} = E$ is commonly termed the optical density.

The numerical value of K depends upon the units in which c and l are expressed. The molecular extinction ϵ coefficient is defined by $\epsilon = E/cl$, where c is in gram moles/l and l in centimetres.

The maximum extinction coefficient of an absorption band may be markedly affected by a change of phase or of solvent but the band area $A = \int K\, d$ often remains constant, giving a better measure of absorption intensity. The band area is related to the oscillator strength.

A fairly frequent analytical problem is the determination of a single constituent after subjecting the sample to physical or chemical treatments to remove interfering materials having absorption. In this case it is first necessary to pick the best wavelength for measurement to be made. This is usually the absorption maximum or a plateau since a small wavelength error makes only a very small error in the intensity determination. It is next necessary to test for adherence to the Beer-Lambert relationship by plotting optical density against concn. If a linear relationship is obtained, comparison of the specific extinction coefficient with that given by a standard pure material enables the concn of material present to be determined.

Occasionally, due to association or other effects, the solutions do not adhere to the Beer-Lambert relationship, i.e., the optical density/concn curve shows marked curvature. In this case the specific extinction coefficient is not independent of concn and measurements must be referred to the prepared curve.

Determination of Two or More Components

When two substances are present in a sample it is usually necessary to measure absorption at two wavelengths in order to determine both materials. The curves of the individual pure substances must also be known. From the curves one wavelength is chosen at which a single component is the sole or major absorber. Knowing the optical densities at the two wavelengths and the extinction coefficients of the pure substances at the two wavelengths the concns of the two substances can be determined.

This may be extended to more than two components by the same principle; a separate wavelength is chosen for each component. In the IR region, because of various complications, a simplified method of calculating concns of single components in a multicomponent system by using the baseline technique has become popular. Typical solvents for the IR region are CCl_4, CS_2 and $CHCl_3$.

The most accurate optical density range for quantitative determinations is from 0.2 to 0.8 and cell-lengths must be chosen to give suitable densities. The theoretical optimum value is 0.4343, which corresponds to 37% transmittance.

Near IR Spectroscopy

In addition to the fundamental region of the spectrum between 2 and 25 μ (5,000 and 400 cm^{-1}) the region from 0.8 to 3.6 μ (12500 to 2780 cm^{-1}) is also useful, particularly for quantitative studies (5,6). Modern automatic recording spectrometers such as the Cary model 14 spectrophotometer have made this region particularly accessible. Whereas bands may overlap in the fundamental region, often separation of the bands now occurs. The bands arise from overtones of fundamental vibrations or are due to combinations of bands. Thus, for example, a band at 1.7 μ (5880 cm^{-1}) is the first overtone of the CH stretching vibration while bands at 2.2 and 2.5 μ (4545 and 4000 cm^{-1}) are combination bands involving the CH stretching with other vibrational modes in the molecule. Quartz cells of known thickness may be used and sample thicknesses, varying from a fraction of a millimeter to 1 or 2 cm, can be examined. This is particularly useful for quantitative studies. Bands arising from *cis* and terminal double bonds are also said to absorb particularly in this region (7). The *cis* double bond has been determined quantitatively using a band (8) at 2.1 μ (4760 cm^{-1}). Hydroperoxides can be studied to advantage in this region. These show absorption bands at 1.46 and 2.07 μ (~6850 and 4830 cm^{-1}) while ordinary peroxides do not show absorption in this region (9).

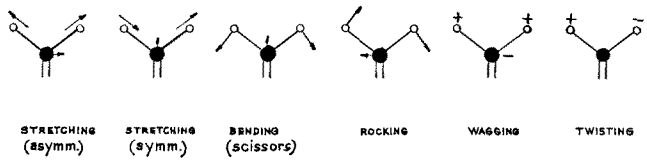


FIG. 1. The various vibrations of the CH_2 group.

Vibrational Spectra of Polymethylene Chains

Many lipids contain polymethylene chains. In order to interpret their spectra we must consider the types of group vibration which couple to produce the vibrations of complete polymethylene chain (10).

The single CH_2 groups of propane have six types of vibration (Fig. 1).

ν CH (as.) CH_2 asymmetric stretching (out of plane)	2926 cm^{-1}
ν (s.) CH_2 symmetric stretching (in plane)	2853 cm^{-1}
δ CH_2 CH_2 bend or scissors	1460 cm^{-1}
ω CH_2 CH_2 wag	1336 cm^{-1}
t CH_2 CH_2 twist	1278 cm^{-1}
ν CH_2 CH_2 rock	748 cm^{-1}

Ignoring vibrations of end groups other vibrations occur. When we have n carbon atoms in the chain we may have

(n–1) C–C stretching modes (in plane)
(n–2) C–C–C angle bending modes (in plane)
(n–3) torsional vibrations about internal C–C bonds (out of plane).

If it is assumed that the polymethylene chain is in the planar *trans* configuration, certain simplifications arise. Because there is a plane of symmetry through the carbon chain the in-plane and out-of-plane vibrations cannot interact. Also the two types of CH_2 stretching vibrations and CH_2 bending vibrations are unlikely to interact strongly. Because the frequency regions of the remaining types of vibrations are well separated, interaction is also expected to be slight. There is good qualitative agreement observed with these predictions and the spectra of paraffins. Half the frequencies are observed to be IR active. Similar distributions have been obtained for other types of vibrations of the polymethylene chain. A number of

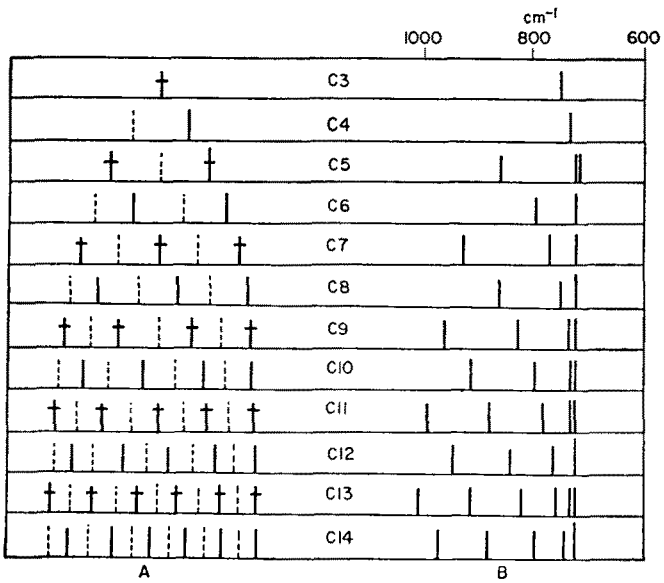


FIG. 2. An idealized distribution of frequencies for CH_2 rocking modes of *n*-paraffins, C_nH_{2n+2} (A), and the experimentally observed IR frequencies (B). (After N. Sheppard.)

factors in practice can effect the symmetry of the observed distributions. The coupling of the CH_2 rocking vibrations is shown both theoretically and experimentally in Figure 2.

Within a particular distribution another selection rule is operative which determines the relative intensities of the spectroscopically active bands. It is expected that as the number of methylene groups increase that the intensity will increase towards either the upper or lower limit of the progression, although this does not always occur. With an infinite chain, however, only those vibrations are spectroscopically active in which every unit cell along the chain vibrates in phase with its neighbours. The unit cell of a regular polymethylene chain consists of two methylene groups. Because of the centre of symmetry within this repeating unit, often one limit of a particular CH_2 distribution is IR active while the other is Raman active.

We now summarise the data obtained on the different types of vibrations:

a) CH_2 stretching and CH_2 bending vibrations

These three classes give rise to narrow frequency regions which usually cannot be separated into separate bands for short chains. With solid state long chain materials the bending frequency at about 6.8 μ (1460 cm^{-1}) is split into a doublet by the operation of interchain forces.

b) CH_2 rocking vibrations

The lower frequency limit of the distribution is well defined and occurs at about 13.9 μ (720 cm^{-1}). The band is split into a doublet with solid state hydrocarbons when the chains are packed in the orthorhombic $\perp$ manner. The high frequency limit of the distribution lies above 9.5 μ (1050 cm^{-1}).

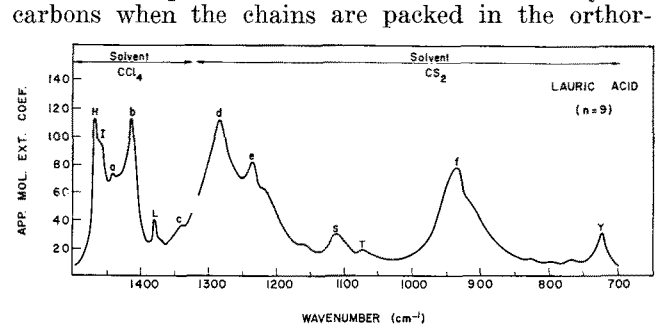


FIG. 3. IR spectrum of lauric acid in solution. (R. N. Jones, *Can. J. Chem. 40*, 321, 1962).

c) CH_2 wagging vibrations

Many long chain compounds show a regular progression of absorption bands in region from about 7.2 to 8.4 μ (1380 to 1180 cm^{-1}). With the alkyl bromides the distribution has been traced to the CH_2 wagging vibration of ethyl bromide. Some discussion has centered upon this distribution as the bands have the same perpendicular polarisation as the CH_2 rocking modes in the spectrum of a long chain monocarboxylic acid. It has been argued that as in the spectrum of an odd-numbered chain of an $X(CH_2)_nX$ molecule, every other CH_2 wagging frequency is predicted to have perpendicular polarisation; it is this sub-set of frequencies which is most enhanced in intensity by interaction with polar end groups. A useful method for calculating the number of CH_2 groups in a long chain molecule of type $X(CH_2)_nY$ is to double the number of observed bands in the CH_2 wagging progression. It is considered that every other band is weak and not usually observable.

d) CH_2 twisting vibrations

There is disagreement about the range of frequencies covered by these vibrations. Analysis of the n-hydrocarbons leads to the range of 7.6 to 8.5 μ (1300 to 1170 cm^{-1}) but 8.3 to 10.7 μ (1200 to 930 cm^{-1}) has also been suggested (11).

e) C–C stretch and CH_3 rocking vibrations

These vibrations are thought to occur in the range 8.6 to 11.0 μ (1150 to 870 cm^{-1}). In many cases, particularly with short chain molecules, there is considerable interaction between them. There is considerable discussion about their assignment (12).

An important matter for the consideration of lipids in liquid crystalline conditions is the extent of nonplanarity of the polymethylene chains. It is important, therefore, to consider how the IR spectrum is affected by internal rotation about any C–C bond. A single gauche configuration about any C–C bond of a polymethylene chain causes a loss of the plane of symmetry present when the chain is in the fully extended all *trans* configuration. Much more interaction is now possible between the different types of group vibrations and therefore complicated spectra are expected. Information about the configurations of the nonplanar isomers of the polymethylene halides has been obtained (13) but there is less information available about the configuration of the methylene chains of lipids.

When spectra are obtained of polymethylene chains in the solid state a number of bands are observed to be split into doublets. This is particularly so when the chains are packed in the orthorhombic O$\perp$ manner. The band at 13.9 μ (720 cm^{-1}) associated with the CH_2 rocking mode is particularly prominent and is split by some 6 cm^{-1}. As many long chain compounds tend to pack in this way the occurrence of a doublet is quite common. With hydrocarbons, as with n-paraffins, the doublet arises from interaction between the two chains present, either in the unit cell or in the sub-cell with long chain derivatives (14,15). When the chains have hexagonal packing (such as esters, acids, etc.) only a single band at 13.9 μ (720 cm^{-1}) is observed. In this form there is effectively only one chain per primitive sub-cell and the interchain distance is increased compared with the orthorhombic O$\perp$ form. With triclinic packed chains T$\parallel$ a single band is observed at 717 cm^{-1} consistent with the occurrence of one chain per unit or sub-cell (14).

Applications

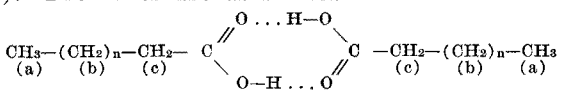
Monocarboxylic Acids. The monocarboxylic acids have been studied to a considerable extent using IR spectroscopy. The vapour, solution, liquid and solid state spectra of many of them have been studied and a great deal of information obtained (16–20). Various topics have been studied including the monomer-dimer relationship (which may occur in the vapour state or in dilute solution), the hydrogen bonding taking place through the carboxyl groups, the polymer formation of the short chain acids in the solid state, and the polymorphism of the longer chain acids. There is such a vast amt of information available that only the main features of the spectral information can be discussed.

Formic and Acetic Acids. We discuss these acids separately from the higher acids since they are somewhat anomalous by comparison although they do have certain features in common.

The IR spectra of formic acid as vapour and in solution have received particular attention (16,21). By contrast with the higher carboxylic acids which are known to be dimeric, the spectrum of formic acid in the liquid has been interpreted to show that in this state the acid is in a polymeric rather than a cyclic dimer form (22). Dielectric polarisation studies indicate a significant contribution from polar configuration (23).

The spectrum of the solid also shows that the acid is in a polymeric form and reveals large crystal splitting consistent with the X-ray studies (22). A complete study of formic acid and deuterated species in the vapour and crystal states has been made and many anomalies in previous assignments resolved (24). However, there are still some uncertainties, concerning the spectra of the crystal. Temp-dependent bands are observed and some fairly large shifts. Some form of polymorphism may occur. In the spectrum of acetic acid the (C = O) band is at a low frequency (25). This is consistent with the polymeric nature of this acid in the crystalline state.

The spectra of monocarboxylic acids have been studied between 6.7 and 15.4 μ (1500–650 cm^{-1}) in carbon tetrachloride and carbon disulfide solution and discussed using a concept of characteristic zones (26). The zones are as shown.



Using this zone concept the following assignments of the carboxylic acid spectrum are made. The zones are marked in the spectrum of lauric acid shown in Figure 3.

Zone a. Terminal methyl group.

A band L is identified with the terminal methyl group and to the symmetrical CH deformation. The peak frequency is displaced progressively from 1385 to 1378 cm^{-1} as the chain length increases. This may arise from weak coupling with a skeletal mode.

Zone b. The polymethylene chain.

A band Y is associated with the CH_2 out-of-plane rocking mode and shifts progressively to 13.9 μ (721 cm^{-1}) with chain length. A band S shifts from 9.3 to 9.0 μ (1086 to 1115 cm^{-1}) for n $\geqslant$ 13 while a band T appears as an inflection for n = 6,11,15–18 and is resolved for n = 7,9,13,14. Its position is similar to the methyl esters and occurs near 9.5 μ (1050 cm^{-1}) for n = 6. Other weak inflections in this region are associated with skeletal modes of various subunits of the chain in *trans-trans trans, gauche* and *gauche-gauche* conformations. The intensity data is very similar for the Y bands. The intensities of the S bands are not much affected by chain length.

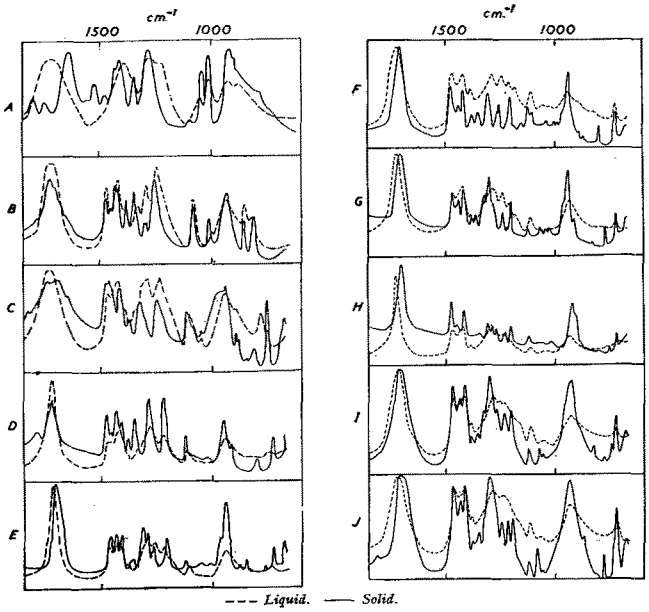


FIG. 4. IR spectrum of monocarboxylic acids, liquid and solid. Acids: *A*, acetic; *B*, propionic; *C*, butyric; *D*, valeric; *E*, hexanoic; *F*, heptanoic; *G*, octanoic; *H*, nonanoic; *I*, decanoic; *J*, dodecanoic. (Corish, P. J. and D. Chapman, *J. Chem. Soc.* 1746, 1957.)

Zone c. A band at 7.5 μ (1336 cm^{-1}) is associated with a wagging mode of the α-methylene group.

Band a) This may be identified with a scissoring vibration of the α-methylene group.

Band b) A band occurs near 7.0 μ (1416 cm^{-1}) and is associated with the carboxylic dimer group.

Band c) This is possibly to be associated with the α-methylene wag.

Band d) and e) This is a doublet probably associated with C–O stretching and OH in plane deformation coupled vibrations. The position of band d shifts in an unsystematic manner between 7.9 and 7.8 μ (1276 and 1288 cm^{-1}) for the short chain compounds, for n $\geqslant$ 9 it remains steady at 1281 $\pm$ 1 cm^{-1}. The weak band e) is more variable in position but becomes steady at n $\geqslant$ 9. The contour of the band is modulated in a characteristic way for each acid, and this may arise from coupled wagging and twisting vibrations of the methylene chain units. It may indicate that the polymethylene chains are predominantly in the *trans* configuration even in solution.

Band f) This band is an out-of-plane deformation mode of the carboxylic acid dimer ring and it exhibits a pronounced asymmetry on the low frequency side.

The intermediate chain length acids are interesting since they are all thought to occur in hydrogen bonded dimeric ring forms while the crystallisation of the acids, at this chain length, is not completely dominated by the hydrocarbon chains The spectra of these acids have been examined in solution (27,28) in the liquid and in the solid state (29). Spectra of the acids in the liquid and solid state are shown in Figure 4.

The dimeric carboxyl group gives rise to absorption bands at 1420 $\pm$ 20, 1300 $\pm$ 15 and 935 $\pm$ 15 cm^{-1}. The first two bands correspond to closely coupled OH deformation and C–O stretching vibrations in the plane of the dimeric ring and the third band is caused by the out-of-plane OH deformation mode. A

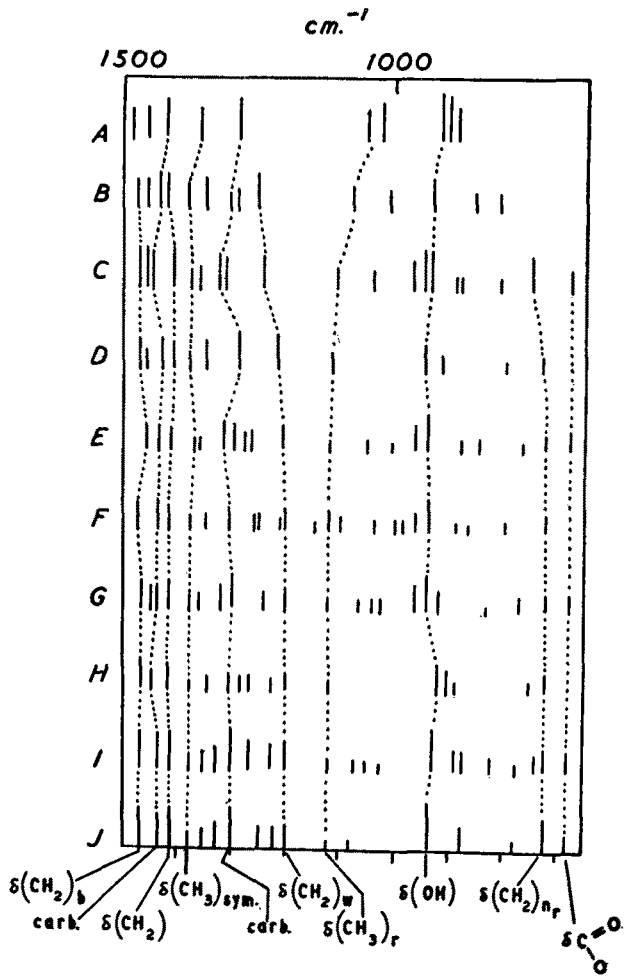
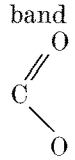


FIG. 5. Assignments of bands of particular vibrations for a series of monocarboxylic acids. (Corish, P. J. and D. Chapman, *J. Chem. Soc.* 1746, 1957.)

band near 14.7 μ (680 cm^{-1}) is associated with a



deformation mode. The assignments are shown in Figure 5.

Comparison between the spectra for the liquid and crystalline states of these acids (Table I) show that while in general crystallisation results in only small shifts in the C = O frequencies, the region between 7.4 and 14.2 μ (1350 and 700 cm^{-1}) becomes much less defined for the liquid state. It is suggested that this latter effect is due to an increase in the number of possible rotational isomers in the liquid state, but it may be that thermal agitation in the liquid tends to make the twisting and the wagging modes of the additional CH_2 groups less well defined.

Slight shifts and additional bands in the 4.0–3.3 μ (2500–3000 cm^{-1})–OH region (OH stretching) also result from crystallisation. These shifts are thought to be due to perturbation effects of the crystalline field or to small changes in hydrogen-bond distances. The additional bands in the 3.3 μ (3000 cm^{-1}) region are considered to arise from combination of the OH stretching frequency with lattice vibrations or with low frequencies associated with the dimeric ring. In the spectrum of hexanoic acid these bands are at approximately constant frequency of ca. 60 cm^{-1}. An alternative explanation for the extra peaks is in terms of summation bands due to appropriate combinations of fundamental vibrations of the coupled carboxyl groups of the dimeric molecules. Series of bands in the 11.1–13.9 μ (900–720 cm^{-1}) region of the spectra of the acids are attributed to CH_2 rocking modes. The most characteristic series of bands in the 13.9–12.3 μ (720–810 cm^{-1}) region is listed showing the corresponding CH_2 rocking frequencies for normal paraffins in the crystalline state and for dicarboxylic acids. It appears that probably all of these acids crystallise with the hydrocarbon chains predominantly in trans zig-zag configuration.

TABLE I

Acid	C=O (cm^{-1}) Liquid	Crystalline (cm^{-1})	
Acetic	1715	1654	
Propionic	1725	1730, 1710(sh)	
Butyric	1729	1740, 1705	
Hexanoic	1721	1710	
Heptanoic	1724	1710	−14
Octanoic	1722	1702	−20
Decanoic	1716	1705	−11
Dodecanoic	1720	1705	−15

TABLE II

Frequencies (cm^{-1}) assigned to $\delta[CH_2]_n$ rocking vibrations

n	Monocarboxylic-acids $H \cdot [CH_2H_2]n \cdot CO_2H$	Dicarboxylic acids $HO_2C \cdot [CH_2]_n - CO_2H$	Paraffins $H \cdot [CH_2]_n \cdot H$
2	807	804	822
3	750	754	748
4	731	733	732
5	727	731	728
6	724	725	726
7	723	726	723
8	723	722	722
9	721	.....	720
11	721		720

As the chain length of the monocarboxylic acids increase so does the number of bands in the 7.2–8.5 μ (1380–1180 cm^{-1}) region (Table II).

The long chain acids are particularly interesting because of their solid state behaviour and their spectra can be particularly informative. Long chain acids such as lauric and higher acids show a regular series of bands in the 7.4–8.5 μ (1350–1180 cm^{-1}) region and the number and appearance of the bands can give information on the length of the chain involved. Lauric acid shows only three regularly spaced bands, the lowest frequency band is at 8.4 μ (1195 cm^{-1}). With increase in chain length the number of bands increases (Fig. 6). The number of bands increases by one for every two methylene groups in the length of the chain. With an even-acid the number of bands in the progression = $^n/_2$, while with an odd-acid the number in the progression = $^n/_2 + \frac{1}{2}$. Similar bands are shown by long chain esters, paraffins and glycerides and are associated with CH_2 wagging modes (30,33). The virtual disappearance of the bands in the molten acids has been suggested to be due to a continuous and random distribution of the $(CH_2)_n$ chains.

The long chain acids exhibit polymorphism and spectral differences arise because of this (34,37). The even-membered acids occur in modifications A, B and C giving different long spacings decreasing in that order. The polymorphism of the odd-membered acids is more complex, the C′-form is always the first to separate from the molten acid and this changes some 10 or 20C below the solidifying point into one or both of the forms A′ and B′. The IR spectra of the even-acids show quite marked changes for the different polymorphic forms.

Spectral changes with temp are observed for tridecanoic and pentadecanoic acids (37) (Fig. 7). With the tridecanoic acid in its most stable A′-form only a single band is observed at 716 cm^{-1} consistent with the triclinic T|| packing of the chains in this form. With pentadecanoic acid in its most stable

A′-form there is a single band at 716 cm^{-1} for the A′-form which changes to a doublet at 727 and 719 cm^{-1} consistent with the orthorhombic packing of this B′-form. This finally changes to a single band at 13.9 μ (720 cm^{-1}) consistent with the near-hexagonal character of the C′-form near its melting point. The variation in the appearance of the bands in the 7.2–8.5 μ (1380–1180 cm^{-1}) region with the C′-form is thought to be related to the flexing and molecular mobility of the hydrocarbon chains which occur in this form. The appearance of the spectrum is analogous to that observed with other long chain compounds such as the 1-monoglycerides and ethyl esters when in their hexagonal or α-crystalline form. It is in contrast to that observed with anhydrous sodium soaps where all the fine detail in this region disappears some 100C below the melting point. The spectra of the even acids, e.g., stearic acid in its C-form show only a very slight shift and fall in intensity in the high frequency component of the 13.9 μ (720 cm^{-1}) doublet. The lower chain length acids show a little more spectral change somewhat analogous to that observed with the odd-acids (cf capric acid).

Polarisation data have been obtained with the various polymorphic forms. This data helps to augment the X-ray data on the structure of the acids in these forms (38,39). One difficulty, however, in the application of the method arises with the triclinic crystals. This is because there are no rules for polarisation of the absorption bands with such crystals. Stearic acid and vaccenic acid have been studied in this way. The latter has been studied and, by means of a reflecting microscope attachment and polarised radiation, is considered to crystallise in an orthorhombic system with the hydrocarbon chains packed into an orthorhombic O⊥ sub-cell similar to polyethylene and the C-form of the saturated acids. A new polymorphic form is claimed to have been observed with octadecanoic acid (40).

Unsaturated Acids

The unsaturated acids are particularly important components of biological materials. Certain features of the spectra are similar to those of the saturated acids, i.e., bands associated with the methylene group of the chain, the terminal methyl group and the carboxy and carbomethoxy groups are similar to those of the saturated acids and methyl esters. Certain bands are, however, associated with the ethylenic group. These occur in the region 3.3–3.2 μ (3000–3100 cm^{-1}), 6.3–6.1 μ (1580–1650 cm^{-1}) and 10.2–14.5 μ (980–690 cm^{-1}) corresponding to stretching vibrations of the C = C–H group, stretching vibrations of C = C bonds and out-of-plane bending vibrations of the C = C–H carbon hydrogen bond, respectively.

The possibility of using the spectra for analytical applications has been discussed. In the C–H stretching region a band at 3.3 μ (3020 cm^{-1}) increases with the number of *cis*-double bonds present, while the relative intensity of the methylene peak at 3.4 μ (2920 cm^{-1}) falls. These observations provide a basis for the evaluation of the degree of unsaturation. If d_A is the optical density at 3.3 μ (3020 cm^{-1}) and d_B the optical density at 3.4 μ (2920 cm^{-1}) a plot of $d_B/(d_B-d_A)$ against the number of double bonds is approximately linear for oleic, linoleic, linolenic and arachidonic acids, while stearic and palmitic acids also fall on the same curve. Analytical working curves can be established for suitable mixtures of saturated and *cis*-unsaturated acids, and as the measurements are based on determinations of intensity ratios the sample concn or sample thickness is not required. A method based on this principle, in conjunction with an IR microscope, could permit the determination of the

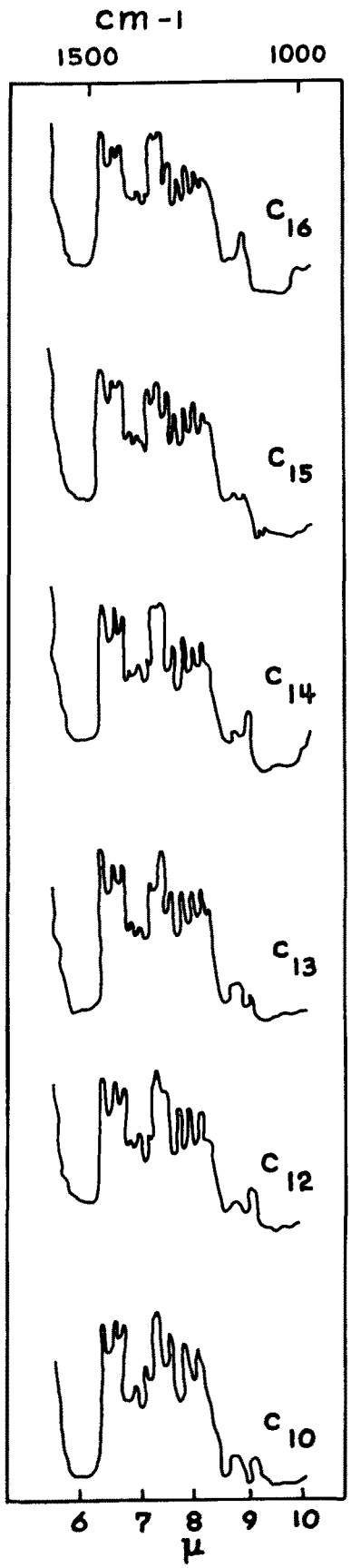


FIG. 6. Spectra of normal aliphatic acids in potassium bromide matrix showing the build up of a regular series of bands. (Meiklejohn, R. A. *et al.*, *Anal. Chem. 29*, 329, 1957.)

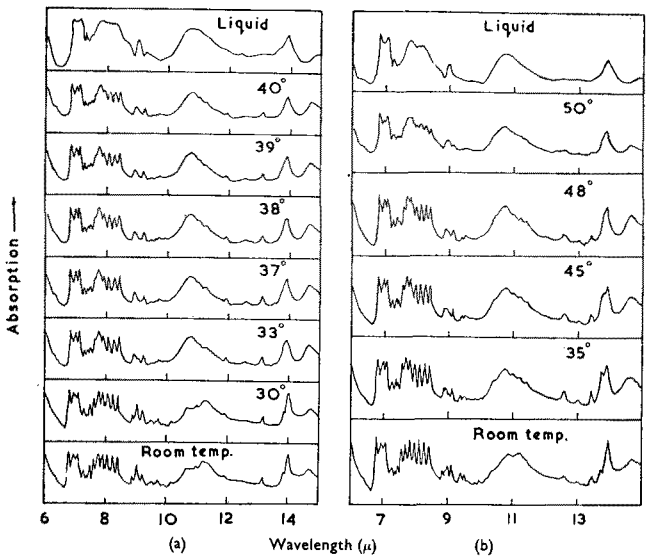


FIG. 7. IR spectral variations with temp (*a*) for tridecanoic acid, and for (*b*) pentadecanoic acid. (Chapman, D., *J. Chem. Soc.* 2310, 1962.)

degree of unsaturation of suitable fatty acid mixtures on microgram amts. Divergencies from the linearity of the analytical working curve may be expected if the chain length varies considerably among the samples or if the acid mixtures contain appreciable quantities of *trans*-ethylenic linkages. Palmitic and stearic acids give almost coincident points so small changes in chain length do not appear to affect the results seriously.

The problem of the identification and estimation of individual unsaturated acids in mixtures is more difficult. The spectra of oleic, linoleic and linolenic acids are very similar, but it is possible, in sclution and in liquid films, to obtain working curves for the analysis of mixtures of linoleic and linolenic acids from measurements of the ratios of the optical densities of the peaks at 9.0 μ (1120 cm^{-1}) in linoleic and 9.4 μ (1067 cm^{-1}) in linolenic acid. Small differences can also be observed between the liquid phase spectra of oleic and linoleic acids and between methyl linoleate and methyl linolenate but these bands are all of low intensity and unsuitable for the analysis of mixtures.

The analysis of binary mixtures of unsaturated fatty acids may be possible using solid phase low temp spectra. The spectra of oleic and linoleic acids differ considerably, especially between 15.1 and 13.1 μ (660 and 760 cm^{-1}).

It is suggested that mixtures of oleic and linoleic acids might be analyzed by matching the solid phase spectra with an atlas of the spectra of standard mixtures. Complications due to polymorphism may occur and should be watched carefully.

Ethylenic CH Bending Vibration

A strong band at 10.3–10.2 μ (965–975 cm^{-1}) is associated with the *trans* substituted ethylene structure and is used to differentiate between *cis* and *trans* substituted unsaturated fatty acids and esters. The band occurs in elaidic acid. A band near 14.4 μ (690 cm^{-1}) has been associated with the vibration of the *cis* grouping but this is less certain.

TABLE III

	Wavelength μ	Wave number cm^{-1}
Isolated *trans*	10.34	967
cis,trans (conj.)	10.17	983
trans,trans (conj.)	10.12	988
cis,cis,trans (conj.)	10.11	989
cis,trans,trans (conj.)	10.09	991
trans,trans,trans (conj.)	10.06	994

A band at 7.0 μ (1435 cm^{-1}) increases in intensity with the amt of unsaturation and has been tentatively assigned to a bending vibration of a methylene group adjacent to a double bond.

A great deal of work has taken place to provide a reliable method for estimating the amt of isolated *trans* content and the American Oil Chemists Society have a tentative official method. The procedure applies to fatty acids if they contain 15% or more *trans* content. If the *trans* content is below this, the fatty acid is converted to the methyl ester. This eliminates overlapping absorption from the nearby carboxylic band.

The quantitative determination of *cis, trans* and *trans,trans* unsaturation has also been described. The *cis,trans* compounds in the presence or absence of *trans,trans* conjugation is determined directly by absorption at 10.55 μ (952 cm^{-1}). The *trans,trans* content is estimated directly from the absorption at 10.11 μ (990 cm^{-1}) unless *cis,trans* isomers are present when correction is needed for absorption due to this grouping.

A strong band at 10.4–10.3 μ (965–975 cm^{-1}) was first associated with a *trans* grouping in 1947 and later shown to arise from a CH deformation about a *trans* C = C group. Different substituents about the double bond give rise to characteristic absorptions.

Conjugation of the unsaturated group is also characteristic. Thus *trans,trans* groupings are characterised by a band at 10.11 μ (990 cm^{-1}) and *cis,trans* groupings by a doublet at 10.18 and 10.55 μ (990 and 952 cm^{-1}). These correlations are given in Table III.

The frequency of bands arising from the conjugation of a *cis* group has also been calculated and correlated with experimental data: *cis* 10.95 μ (913 cm^{-1}), *cis,cis* (conj.) 10.7 μ (934 cm^{-1}), *trans,cis* (conj. 10.53 μ (950 cm^{-1}). [In this case two bands occur.] All these bands are weak or very weak in intensity. The determination of *cis* absorption appears to be better carried out using the near IR region.

The spectra of the solid state unsaturated acids in the 7.4–8.4 μ (1350–1180 cm^{-1}) region are informative. Whereas with the saturated acids a regular series of bands occur, for the *cis*-unsaturated acids the progression becomes irregular (41). With the *trans* acids however the region is more similar to the saturated acids, but here the band progression depends upon the chain segment next to the carboxyl group. This has been nicely illustrated by an examination of a number of octadecenoic acids with *trans* double bonds in positions varying from the 6- through to the 11-position (42) (Fig. 8). The bands can be contrasted with the same region for the saturated acids from C_6 to C_{12}. Apparently the terminal chain segments do not give rise to regularly spaced medium intensity bands. However, some weak bands occur between the main progression bands which might be caused by the terminal segments.

Polymorphism does not occur with these acids but this might be a complication in the application of this method to the determination of the position of the double bond in other acids. The frequency limit for the series is affected with these acids and the weak bands in the 7.4–7.6 μ (1350–1300 cm^{-1}) region are excluded because the spacings are irregular; positions do not change with chain length, and the polarisation characteristics are different.

The hydroxyl groups of the hydroxy acids cause the packing of the molecules to be different from that observed with the normal acids. Hydrogen bonding also occurs. Susi has examined a number of dihydroxy acids (42). All the acids show strong absorption around 2.9 μ (3,400 cm^{-1}) and a series of bands of medium strength between 10.0 and 8.3 μ (1000 and 1200 cm^{-1}) corresponding to the OH stretching vibrations and the C–O stretching and/or deformation vibrations, respectively. Only weak bands occur in the 8.4–7.0 μ (1180–1350 cm^{-1}) region with no apparent regularity. Whilst the position isomers of contiguously substituted dihydroxy stearic acids cannot be identified by clear-cut regularities as with the *trans* monounsaturated acids there is enough spectral detail in the region between 8.3 and 12.5 μ (1200 and 800 cm^{-1}) to allow identification on a fingerprint basis. The high melting and low melting acids are distinguished in the 10.0 and 8.6 μ (1000 and 1150 cm^{-1}) region by the more complex structure of the low melting members.

Branched Chain Acids

A series of monomethyl-substituted octadecanoic acids has been studied in the solid state (43,44). In the 7.5 to 8.4 μ (1330 to 1180 cm^{-1}) region as the methyl branch is moved towards the carboxyl group irregularities are seen as many inflections in the band progression. Unusual sub-cells occur with these acids and crossed chains are also thought to occur.

Esters

The esters have been studied extensively in solution (28,44), as liquids and in the solid state and yet there are still uncertainties concerning the assignments of some bands in their spectra (28,45,46).

Particular features of the spectrum of the esters are the bands associated with the carbonyl C = O groups. The carbonyl frequencies of a large number of simple esters in the liquid condition lie in the range 1750–1735 cm^{-1}. This enables the esters to be distinguished from the normal ketones as the carbonyl frequency is raised in the esters by the influence of the neighbouring oxygen atom. Jones and co-workers (47) have found that the carbonyl frequencies of over a hundred sterol acetates, propionates, etc., occur in the region 1742–1735 cm^{-1} regardless of the position of the ester group in the steroid nucleus.

The influence of $\alpha\beta$ unsaturation is to lower the carbonyl frequency while $\gamma\delta$ substitution has little if any effect. Conjugation with acetylenic links produce a greater shift to the range 1720–1708 cm^{-1}. With vinyl esters (CO–O–C = C) a marked enhancement of carbonyl frequency occurs. Thus vinyl acetate absorbs at 5.6 μ (1776 cm^{-1}). In α-diesters and α-keto esters the degree of interaction of the two adjacent CO groups is apparently very small. Some β-keto esters can undergo enolisation. Ethyl-α-methylacetoacetate and ethylacetoacetate show an additional band at 6.0 μ (1650 cm^{-1}) which is attributed to the ester carbonyl group after chelation to the enolic hydroxyl group.

The C–O stretching mode gives rise to strong bands in the 7.6–10.0 μ (1300–1000 cm^{-1}) region in many compounds. Its precise frequency is sensitive to changes in the mass and the nature of the attached groups. The formates have a strong band in the 8.0–9.5 μ (1250–1050 cm^{-1}) region, with methyl formate it is at 8.2 μ (1214 cm^{-1}), ethyl formate at 8.4 μ (1195 cm^{-1}) and becoming 1185 cm^{-1} in the higher homologues. The acetates show a strong band in the range 1250–1230 cm^{-1} and it is near 1245 cm^{-1} in the small chain length acetates. A second but weak absorption occurs in the 9.4–10.0 μ (1060–1000 cm^{-1}) range. The frequencies of the CO stretching mode of the series of simpler higher esters within each group are fairly constant. They are listed in Table IV.

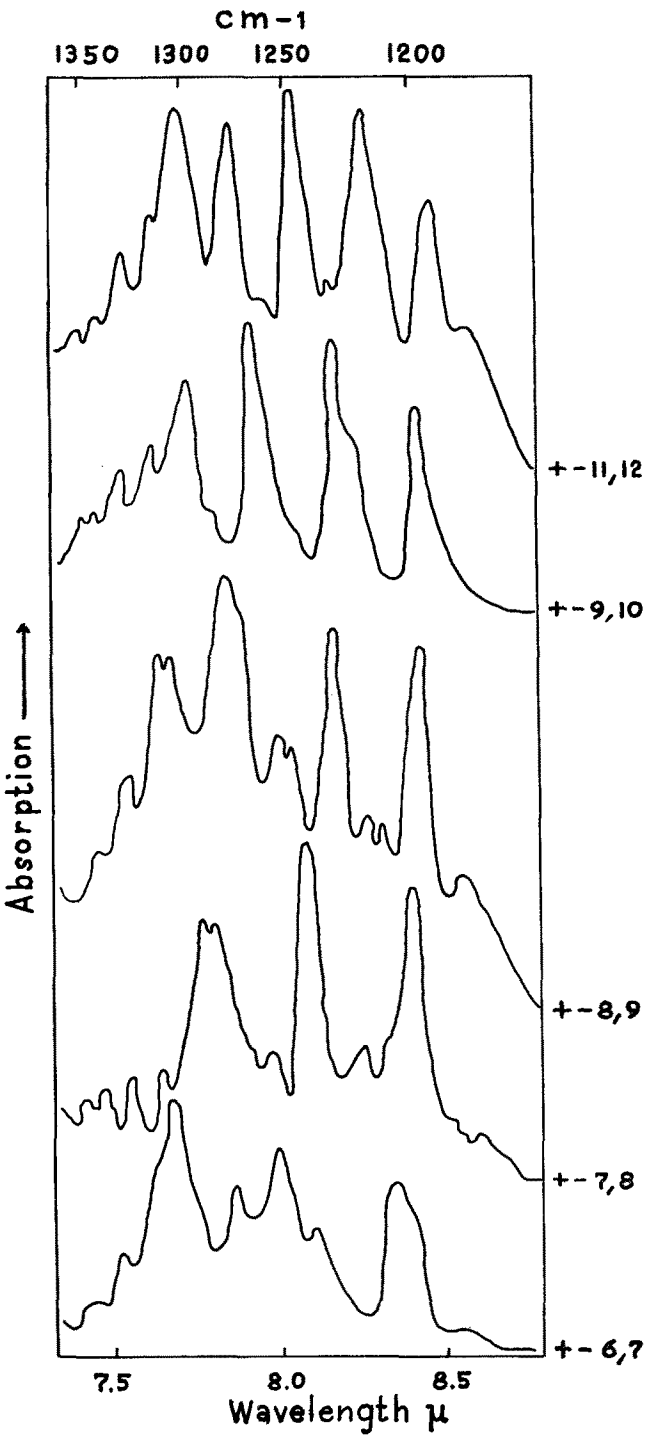


FIG. 8. 1175–1350 cm^{-1} region of transoctadecenoic acids. (Susi, H. *Anal. Chem. 31*, 910, 1959.)

Useful correlations for the spectra of a large number of aliphatic esters in the 15 to 40 μ (670–250 cm^{-1}) region have been published (48).

In an analogous manner to the carboxylic acids the zone concept has been applied to the solution spectra of the methyl esters (26). Four vibrational zones are discussed as shown

$$\underset{(a)}{CH_3} - \underset{(b)}{(CH_2)_n} - \underset{(c)}{CH_2} - \underset{(d)}{COOCH_3}$$

TABLE IV

Class	C-O stretching frequency
Formates	1200–1180 cm^{-1}
Acetates	1250–1230 cm^{-1}
Propionates	1200–1150 cm^{-1}
Butyrates	1190 cm^{-1}
Isobutyrates	1200 cm^{-1}
Adipates	1175 cm^{-1}
Laurates	1163 cm^{-1}
Oleates	1172 cm^{-1}
Ricinoleate	1174 cm^{-1}
Stearate	1174 cm^{-1}
Sebacates	1172 cm^{-1}

Bonds are marked in an analogous way to those of the carboxylic acids. Those marked H,I,L,S,T and Y are correlated with their counterparts in the spectra of the esters.

By using deuterated derivatives a detailed assignment of methyl laurate has been obtained (49) and is given in the Table V.

It has been noted that the band at 13.9 μ (720 cm^{-1}) requires the presence of at least four *trans* linked methylene groups. As this band increases linearly with chain length for many polymethylene compounds it has been inferred that in the liquid state the chains exist predominantly in conformation sets in which at least consecutive methylenes are linked in a *trans* grouping. Each section will then contribute a set of bands to the vibrational pattern determined by its chain length. The super-position of bands from the units of different length could produce the broad band system observed. The IR and Raman spectrum of polymethylene compounds in the liquid state is said to agree in the main rather well with an assignment based on a vibrational analysis of the all *trans* methylene chain.

The spectra of a large number of ethyl esters have been examined in solution, liquid and the solid state (28,33).

The ethyl esters have a band at 9.6 μ (1042 cm^{-1}) which is not observed with the methyl esters and enables them to be distinguished. The long chain ethyl esters exhibit polymorphism and the IR spectra of the different forms have been studied.

TABLE V

Summary of Band Assignments for Methyl Laurate

Band [a]	Max	Max [a]	Assignment	
			Group	
A	[3020] cm^{-1}	16	$-COOCH_3$	Asym. C – H stretch (a′)
B	2995	40	$-COOCH_3$	Asym. C – H stretch (a″)
α	2955	....	$-COOCH_3$	Sym. C – H stretch
C	2950	260	CH_3-	Asym. C – H stretch
D	2922	520	$-(CH_2)_9-$	Asym. C – H stretch
E	[2870]	120	CH_3-	Sym. C – H stretch
F	2852	290	$-(CH_2)_9-$	Sym. C – H stretch
G	1742	550	$-COOCH_3$	C = O stretch
H	1465	113) Band β) Band γ	$-(CH_2)_9-$ CH_3-	C – H scissor Asym. C – H bend
I	1458	107) Band δ	$-COOCH_3$	Asym. C – H bend (a″)
ϵ	1440	....	$-(CH_2)_9-$	C – H scissor or wag
J	1436	135	$-COOCH_3$	Sym. C – H bend
K	1419	36	$\alpha-CH_2-$	C – H scissor
L	1378	51	CH_3-	Sym. C – H bend
	1368	....	$-(CH_2)_9-$	C – H wag or twist
M	1362	62	$\alpha-CH_2-$	C – H wag
η	1352	....	$-(CH_2)_9-$	C – H wag or twist
N	[1340]	40	$-(CH_2)_9-$	C – H wag or twist
O	1305	50	$-(CO_2)_9-$	C – H twist (or wag?)
P	1248	97		
Q	1196	135	$-CH_2$ $COOCH_3$	C – C skeletal coupled with $\alpha-CH_2-$ deformation
R	1169	162	$-CH_2$ $COOCH_3$	C – C skeletal coupled with $\alpha-CH_2-$ deformation
S	1112	64	$-CH_2$ $COOCH_3$	C – C skeletal coupled with $\alpha-CH_2-$ deformation
T	1074	25	$-(CH_2)_9-$	C – C skeletal coupled with end groups
U	1015	33	..... $-CH_2$ $COOCH_3$	C – C skeletal C – C skeletal coupled with $\alpha-CH_2-$ deformation
V	875	....	$-COOCH_3$	Methyl rock ?
W	845	....	$-COOCH_3$	Methyl rock ?
X	755	8	$-(CH_2)_9-$	C – H rock
Y	722	27	$-(CH_2)_9-$	C – H rock

[a] Points of inflection are designated by square brackets. Greek letters identify bands that are not observed in the methyl laurate spectrum but are presumed to be present from the analysis of the spectrum of the deuterated derivatives.

Alcohols

A considerable number of studies of alcohols have been published dealing with various aspects of their spectra. The band arising from the OH group and its variation of position and intensity as a function of concn and change of state has received particular attention (50,51). The frequency range for the unbonded OH stretching vibration is 3650–3590 cm^{-1}, but this shifts on hydrogen bonding with intermolecular hydrogen bonds to 3550–3450 cm^{-1} for single bridge compounds and 3400–3200 when polymeric association occurs.

Absorption bands in the low frequency region of the spectrum arise from C–O stretching and from the OH deformation mode. Two bands occur covering a wide frequency range which may be associated with these vibrations but both are affected by hydrogen bonding. Coupling effects make it difficult to assign the bands. The range of absorption of these bands are given below.

Primary alcohol	~1050 cm^{-1} (s)	1350–1260 cm^{-1} (s)
Secondary alcohol	~1100 cm^{-1} (s)	1350–1260 cm^{-1} (s)
Tertiary alcohols	1150 cm^{-1} (s)	1410–1310 cm^{-1} (s)

The spectra of a number of long chain alcohols of primary, secondary and tertiary in the solid state have been examined (52). The regular series of bands arising from the polymethlene chain in the 8.4 to 7.2 μ (1190 to 1380 cm^{-1}) region are observed. With the primary alcohols the spacings are given by $\Delta n = 19 \pm 1$ cm^{-1}.

The primary alcohols show a doublet in the 13.9 μ (720 cm^{-1}) region while the secondary alcohols show only a single band. This is presumably due to a difference in the mode of hydrocarbon packing in the two cases.

The polymorphism of some long chain alcohols has been studied using IR spectroscopy (Fig. 9). By rapid scanning of the region near 13.9 μ (720 cm^{-1}) as the temp was varied the transitions from one form to another have been followed (33).

Anhydrous Soaps

Several changes of state occur when an anhydrous sodium soap is heated from room temp (53,55) to near the melting point, e.g. dilatometric measurements over this temp range (54) show two gross irregularities in the density-temp curve at about 105–120C and 200C, with minor breaks at other temps. With anhydrous sodium palmitate, five phase transitions have been observed between the crystalline state and the isotropic melt. An X-ray examination of this soap (55) shows that the long spacings vary with temp but suggests that the phases can be grouped into two basic structures, named the waxy and neat phase, both being thought to be liquid-crystalline.

The IR spectrum of sodium palmitate at different temps is shown in Figure 10.

As the temp approaches 100C the distribution of bands in the 8.0 μ (1250 cm^{-1}) region becomes less well-resolved (56), particularly the bands at the high-frequency end of the distribution. This is interpreted to mean that the hydrocarbon chains are beginning to flex and twist. (This will produce some rotational

isomerism, each isomer having its own frequency in this region, producing a smearing-out of the spectrum.) This interpretation is supported by the fact that the band at 13.9 μ (719 cm^{-1}) also decreases slightly in intensity. (This band is associated with the in-phase motion of all the CH_2 groups, so that twisting of the chain will reduce the intensity of this band.) On further heating to about 120–130C this band decreases considerably in intensity, indicating that many more methylene groups are spinning freely about the C–C bonds. All the fine structure in the 8.0 μ (1250 cm^{-1}) region vanishes, while bands assigned to other CH_2 rocking modes also become diffuse or disappear. Bands assigned to methyl rocking modes decrease in intensity but remain prominent. There is little if any movement in frequency of the carboxylate bands at 6.4 μ (1560 cm^{-1}), 7.1 μ (1415 cm^{-1}) or 14.3 μ (695 cm^{-1}) but the intensity of the 14.3 μ (695 cm^{-1}) band decreases.

Above 120–130C the spectrum of the soap is in fact entirely "liquid-like" in character. The changes observed in the spectra are analogous to those observed in the spectra of, e.g., long-chain monocarboxylic acids when the transition from the crystalline to liquid state occurs. Yet this transition occurs some 160C below the "true" melting point of the soap. It seems clear that at this temp the hydrocarbon chains are in a "liquid state." We can conclude that the soap is only prevented from completely melting by the strong bond between the highly polar sodium metal and carboxylate groups. The spectra clearly demonstrate the appearance of the "liquid-crystalline" phase of soaps. X-ray data on this phase support this interpretation, since the long spacings are sharp while the short spacings are diffuse. The spectral evidence showed clearly for the first time that all the phase changes above 110C occur with the hydrocarbon chains in a liquid-like condition.

Little change is observed on further heating of the soap up to 200C, except that the bands at 13.9 μ (719 cm^{-1}) and 14.3 μ (695 cm^{-1}) decrease further in intensity and the relative intensity of the 6.8 μ and 7.1 μ (1460 and 1415 cm^{-1}) band alters. The 1415 cm^{-1} band then becomes of greater intensity.

On cooling of the soap all these spectral changes are observed in reverse order and all the fine detail in the spectra return.

Sodium stearate exhibits analogous spectral changes with change in temp. These changes can obviously be correlated with the variation of other properties of the soaps at these temps, such as flow, yield and mobility (57). The increased cohesion and plasticity and fibre-forming properties observed above 110C are doubtless due to the fluidity of the hydrocarbon chains at this temp.

Glycerides

Glycerides are usually difficult materials to characterize, in the main because of the variety of possible isomers which can occur. IR spectroscopy has however been shown to be a versatile analytical tool with wide applications in this field of investigation. IR spectroscopic work was carried out with the material in solution in either carbon tetrachloride or chloroform. Recent studies have shown that much qualitative information may be obtained, especially about the configuration of glycerides, from the spectra of the materials in the crystalline state. This introduces complications arising from the occurrence of polymorphism, i.e. the existence of more than one crystalline form for the same glyceride. However,

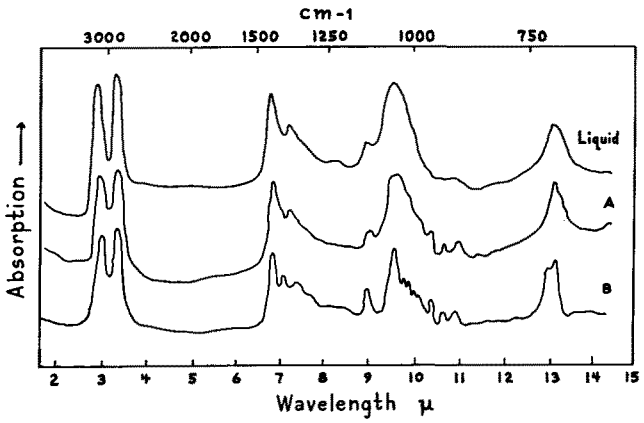


FIG. 9. The polymorphic forms of *n*-cetyl-alcohol.

these complications can be turned to good account, and the spectra may be used to provide information about the crystalline nature of the glyceride.

Triglycerides, diglycerides and monoglycerides have been examined and their spectra are given (58–64).

Triglycerides

O'Connor et al. (65) have given the spectra from 2–12 μ of a number of triglycerides obtained from solutions in chloroform and have assigned the main bands to vibrations of the functional groups present. These bands also occur in the spectra of the liquid. The following table shows the frequencies of the main bands for long-chain triglycerides. The carbonyl band has been used, combined with TLC to provide a quantitative technique for triglycerides in serum lipids. The bands in these spectra are generally broad and smeared into each other.

The spectrum of the randomly oriented crystalline material includes these major bands arising from the functional groups, but splitting of bands and shifts also occur (Table VI).

Saturated Triglycerides

The spectra of the saturated triglycerides are found to vary according to the polymorphic form in which

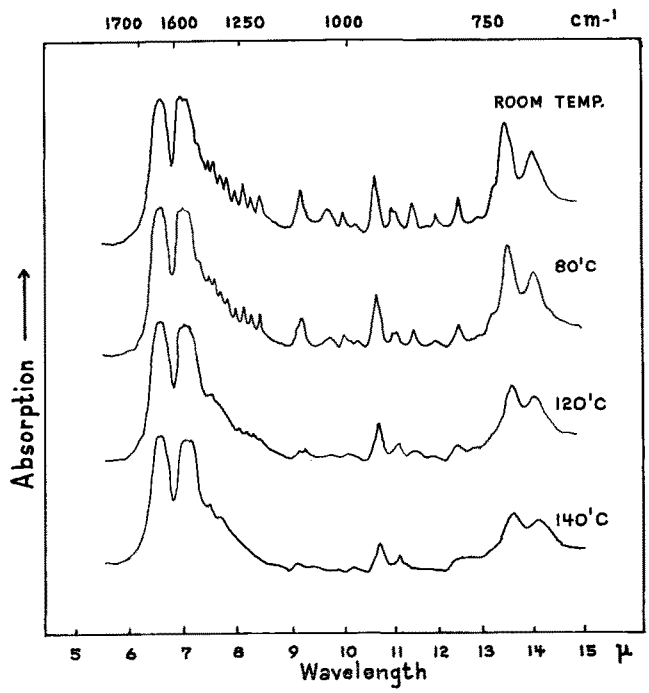


FIG. 10. The IR spectra of anhydrous sodium palmitate at different temps. (Chapman, D., J. Chem. Soc. *784*, 1958.)

TABLE VI

Probable assignment	
C—H stretching (CH_2 and CH_3)	3030–2967 cm^{-1}
C=O stretching (COOR)	1751–1733 cm^{-1}
C—H bending (CH_2/CH_3 groups)	1464–1453 cm^{-1}
C—H bend (symmetrical deform of CH_3)	1383–1361 cm^{-1}
C—H in plane wagging or rocking of CH_2 groups	1261–1250 cm^{-1}
C—O stretching (COOR)	1179–1166 cm^{-1}
CH_2 rocking mode	730–717 cm^{-1}

they occur. The variety of spectra obtained with a single saturated triglyceride in its various polymorphic forms is illustrated in Figure 11 for tristearin. Among other spectral differences a single band occurs at 13.9 μ (720 cm^{-1}) in the spectrum of the α_L-form; a doublet at 719 and 727 cm^{-1} in the spectrum of the β'_L-form; and a single band at 717 cm^{-1} in the spectrum at this temperature. The transition sub $\alpha_L \rightarrow \alpha_L$ rocking mode of the methylene groups). The lowest form, designated sub-α_L, is obtained by quenching the liquid glyceride to −70C and running the spectrum at this temperature. The transition sub-α_L-α_L is observed to be a reversible one. The main CH_2 rocking mode in the spectrum of the sub-α_L form is also a doublet at 719 and 727 cm^{-1}. These variations have been discussed in terms of the packing of the hydrocarbon chains present in the crystals, hexagonal in the α_L-form, orthorhombic O⊥ in the β'_L-form, and triclinic T|| in the β_L-form. The packing of the hydrocarbon chains in the sub-α_L form is also deduced to be probably orthorhomobic on O⊥. For characterization purposes the most suitable form is considered to be the α_L-polymorphic form, which for triglycerides, of greater mol wt than trilaurin, is the form usually obtained by solidification at room temp from the melt. It is therefore fairly easily and conveniently obtained. The spectra of the remaining forms are obtained by suitable thermal treatment or by crystallization from solvent followed by KCl disc or Nujol mull preparation.

The spectra of a series of saturated triglycerides are shown in Figure 12. In each case the glyceride is in the α_L-polymorphic form. The number of bands in the 8.0 μ (1250 cm^{-1}) region increases with increasing chain length as with other long chain derivatives. The frequencies of these bands are given in Table VII. The number of bands for an even number of carbon atoms in the chain is usually equal to half the number of carbon atoms, n, in the chain, i.e. ½n, while for an odd number of carbon atoms it is usually equal to half of the sum of the number of carbon atoms plus one, i.e. ½ (n + 1). While the number of bands in the spectrum of a triglyceride with an odd-numbered chain is the same as in the spectrum of the next higher even-numbered chain, all the bands are shifted in frequency. The frequency difference between the band of highest frequency and its next lower neighbour is always less than the frequency difference between the other bands. These bands are analogous to those observed with anhydrous sodium soaps, fatty acids and esters.

TABLE VII

Saturated triglycerides (α_L-form)	Frequency of bands in the 1250 region (cm^{-1})
Trimyristin	1344, 1329, 1300, 1276, 1252, 1229, 1200
Tripalmitin	1342, 1331, 1308, 1285, 1265, 1240, 1222, 1199
Trimargarin	1334, 1318, 1299, 1276, 1260, 1235, 1219, 1198
Tristearin	1341, 1330, 1308, 1289, 1270, 1255, 1232, 1216, 1193
Trinonadecylin	1346, 1335, 1323, 1302, 1285, 1266, 1248, 1230, 1213, 1194
Tribehenin	1339, 1323, 1310, 1293, 1279, 1265, 1252, 1233, 1222, 1205, 1189
Saturated diglycerides (β_L-form)	**Frequency of bands in the 1250 region (cm^{-1})**
1:3 Dilaurin	1328, 1310, 1298, 1266, 1235, 1211
1:3 Dimyristin	1347, 1329, 1306, 1279, 1258, 1231, 1209
1:3 Dipalmitin	1346, 1332, 1310, 1290, 1266, 1245, 1224, 1205
1:3 Distearin	1340, 1328, 1309, 1292, 1271, 1255, 1231, 1214, 1198
Saturated diglycerides (α_L-form)	**Frequency of bands in the 1250 region (cm^{-1})**
1:2 Dilaurin	1349, 1329, 1299, 1270, 1239, 1210
1:2 Dimyristin	1347, 1333, 1306, 1281, 1259, 1231, 1205
1:2 Dipalmitin	1343, 1331, 1308, 1286, 1265, 1240, 1221, 1199
1:2 Distearin	1342, 1328, 1307, 1290, 1269, 1253, 1232, 1213, 1192
Monoglycerides (β_L-form)	**Frequency of bands in the 1250 region (cm^{-1})**
1-Monostearin	1333, 1294, 1255, 1213
1-Mono-olein	1327, 1283, 1231
1-Monocaprylin	1329, 1293, 1256, 1221
1-Monocaprin	1337, 1302, 1275, 1242, 1215
1-Monolaurin	1332, 1306, 1284, 1258, 1230, 1205
1-Monomyrystin	1339, 1314, 1293, 1271, 1248, 1227, 1202
1-Monopalmitin	1332, 1312, 1297, 1277, 1258, 1233, 1219, 1198

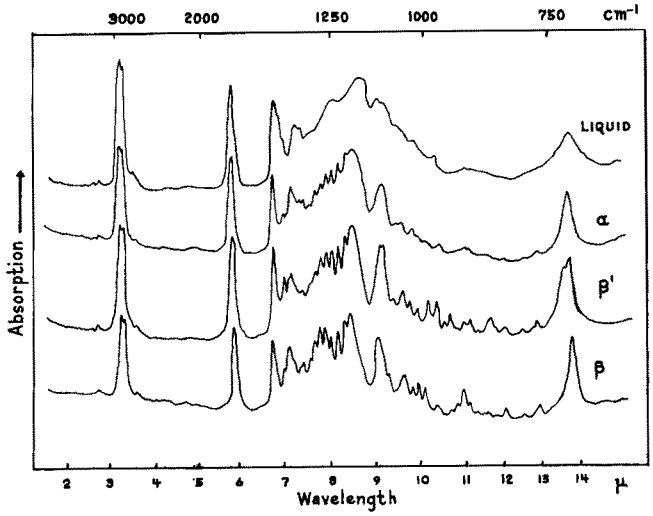


FIG. 11. The polymorphic forms of tristearin.

To identify saturated triglycerides of mixed chain-length is a little more difficult since a number of complications can occur. With glycerides such as 1-palmito-distearin the number of bands in the 8.0 μ (1250 cm^{-1}) region of the α_L-form is the same as that in tristearin while with 1-stearo-dipalmitin the number of bands is the same as in the spectrum of tripalmitin. With some glycerides however, the α_L-form is not stable while with other glycerides the most stable form is a β'_L-form rather than a β_L-form (cf of the spectra of 2-stearo-dipalmitin (PSP) and 2-palmito-distearin (SPS). In the spectrum of a triglyceride of considerable difference in chain length. e.g. 1:2-diaceto-3-palmitin, the band at 7.25 μ (1390 cm^{-1}) is of strong intensity corresponding to the methyl groups present, and the CH_2 rocking mode is a single band at 14.0 μ (718 cm^{-1}), indicating that the hydrocarbon chains are probably triclinically packed in this form as in the β_L-form of other saturated triglycerides.

Unsaturated Triglycerides

The unsaturated triglycerides can be divided into two main types: those containing double bonds with with the *cis* configuration and those with the *trans* configuration. Some glycerides contain both types. There is little difficulty in differentiating these two main types since the presence of the *trans* group is clearly shown by the band at 10.4 μ (963 cm^{-1}). This band occurs in the spectrum of material in the liquid state, in solution, or in the crystalline state. The strong band attributable to the *trans* group in the molecule is apparent in all the spectra. The relative intensities of the bands in the 1250 cm^{-1} region in the spectra change in a manner analogous to that observed with saturated triglycerides.

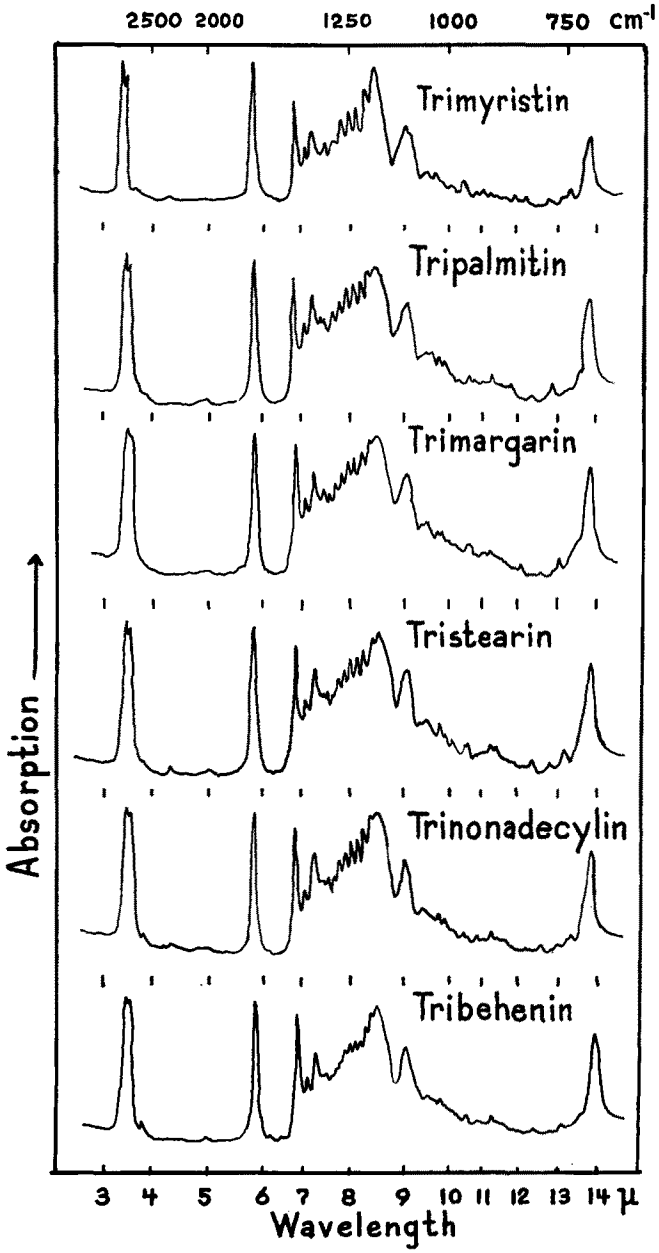


FIG. 12. IR spectra of triglycerides in the α_L-form.

The presence of a *cis* group is usually indicated by the presence of a weak band near 6.0 μ (1660 cm^{-1}). The band attributable to the bending vibration of the hydrogen atoms in the *cis* group is not constant in frequency, however, and is very sensitive to crystal structure. As with the saturated triglycerides a variety of spectra can be obtained, depending on the particular crystalline form in which the material can exist. (Most unsaturated natural lipids exist predominately in the *cis* form but can change to a mixture of *cis* and *trans* forms on partial hydrogenation).

It is preferable, where possible, with these glycerides to obtain more than one spectrum. Examination of the 8.0 μ (1250 cm^{-1}) region of the spectrum of the α_L-form provides information about the chain length. The spectrum of the most stable form, usually obtained by slow crystallization from solvent, is also informative, e.g. the most stable form of 2-oleo type mono-oleo triglycerides crystallizes in a form (14) designated β_L while the most stable form of 1-oleo type mono-oleo triglycerides crystallizes in a form designed β'_L. As a result of this, the spectra of the stable forms of these closely related isomers differ considerably. The spectra of 2-oleo-dipalmitin and 1-oleo-dipalmitin in their most stable forms are shown in Figure 13.

The main CH_2 rocking vibration in the spectrum of the 2-oleo isomer is single at 14.0 μ (717 cm^{-1}) while that of the 1-oleo isomer is a doublet at 729 and 719 cm^{-1}. The spectrum of the 2-oleo isomer also shows a strong band at 690 cm^{-1} attributed to a vibration of the *cis* group. A strong band does not occur in this region of the spectrum of the 1-oleo isomer. A sub-α_L form showing a doublet at 719 and 727 cm^{-1} is also observed with these glycerides. (This is particularly clear in the spectra of 1-oleo distearin at 0C). This large difference observed between the spectra of the 1- and 2-oleo isomers is very useful and enabled us to determine correctly for the first time the major glyceride present in cocoabutter and kokum butter. This has been confirmed by other workers using other techniques. Spectra of other natural fats, such as illipe butter, have also been obtained. The major glyceride in all these natural fats, except lard, appear to have the same 2-oleo type of configuration. Unusual variations may occur when mixed crystal formation occurs. This is particularly observed with mixtures of 2-oleo and 1-oleo distearin. Mixtures of equal amts of these glycerides, crystallized slowly from solvent and dispersed in Nujol, give rise to a spectrum quite different from that of either glyceride and corresponds to a definite mixed crystal formation.

Spectra obtained from the polymorphic forms of triolein are shown in Figure 14. The strong band near 14.4 μ (690 cm^{-1}) and the band at 6.0 μ (1660 cm^{-1}), attributable to the presence of the *cis* group, are apparent. In the spectrum of the α_L-form the band is single at 723 cm^{-1}, and in the spectrum of the $\beta'/_L$-form it is split into two components at 729 and 722 cm^{-1} while in the spectrum of the β_L-form it is single at 722 cm^{-1}. This is closely analogous to the variation of this band observed in the spectrum of the saturated triglycerides. It is suggested that perhaps the chains, or parts of the chains, will pack predominantly in a similar manner to that observed with saturated triglycerides despite the presence of the *cis* groupings in the chain.

Diglycerides

Diglycerides may also exist in two isomeric forms, depending on the position of the chain on the glycerol residue. Thus 1:3 and 1:2 type glycerides occur. Both may also be optically active. Characterization of the two types is usually fairly simple since the

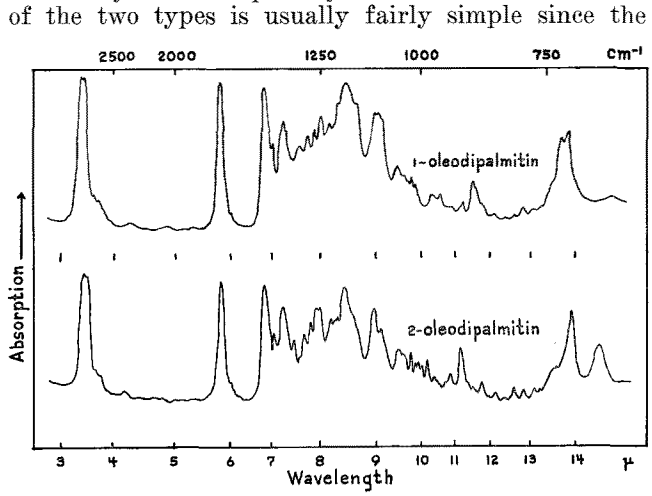


FIG. 13. IR spectra of 1-oleo dipalmitin and 2-oleo dipalmitin.

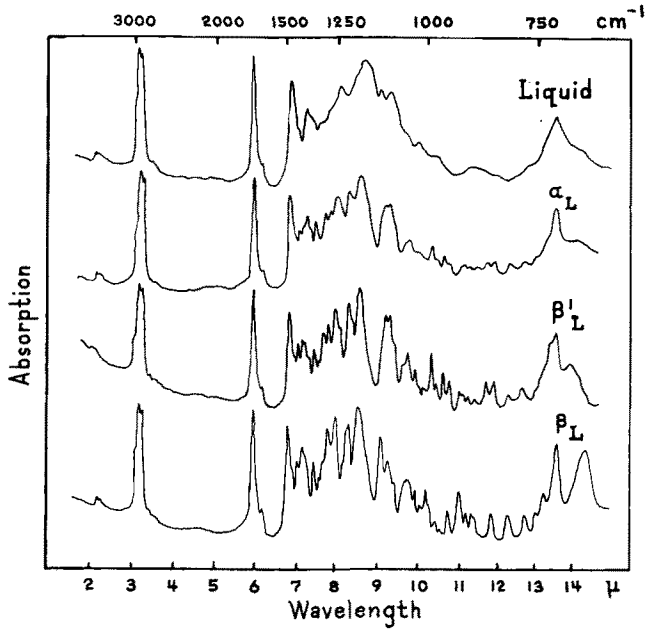


FIG. 14. IR spectra of the polymorphic forms of triolein.

spectra of crystalline forms of these two types of glyceride differ considerably as a consequence of the different modes of packing adopted by the hydrocarbon chains. The 1:3 type of diglyceride, such as distearin and dipalmitin, appears to crystallize in two very similar forms (probably with triclinic packed chains) while the 1:2 type glyceride appears to crystallize in two forms, with X-ray short spacings different in type from those of the 1:3-diglycerides. The spectrum of a diglyceride, such as distearin in its most stable form after crystallizing from solvent and dispersing in Nujol, affords a very convenient way to differentiate between the 1:2 and 1:3 diglyceride isomers. The 1:3 glyceride from the melt gives rise to a spectrum nearly identical to that obtained by dispersing the solvent-crystallized material in Nujol, i.e. it exists in two β_L-type forms. These glycerides do not appear to crystallize in an α_L-form. The spectra of a series of 1:3-diglycerides are shown in Figure 15. Information about the chain length can be deduced from the 1250 cm^{-1} region.

The 1:2 and 1:3 diglycerides have been examined in solution in the fundamental region and the configurations in solution deduced. The diglycerides are considered to have three predominant configurations, one internally hydrogen bonded and two involving no hydrogen bonds. The hydrogen form of the 1:3 diglyceride is considered to have a *trans, gauche* configuration and the other forms a *trans,trans* configuration around the glyceride backbone. With the 1:2 diglyceride the hydrogen bonded structure appears to be in a *trans,trans* configuration. It should be noted that not all 1:3 diglycerides crystallize in the β_L-form ,e.g. 1-aceto-3-palmitin crystallizes in a β'_L-form, and its spectrum is very different from that observed with, say, 1:3-dipalmitin. In particular, the main CH_2 rocking mode near 720 cm^{-1} consists of a doublet.

The spectra of the two polymorphic forms (designated $\alpha_L \equiv \alpha_M$ and $\beta'_L \equiv \beta_M$) of the 1:2 diglycerides have marked spectral differences. In the spectrum of the α_L-form the main CH_2 rocking mode is single at 13.9 μ (720 cm^{-1}), while in the spectrum of the β'_L-form a doublet occurs at 727 cm^{-1} and 719 cm^{-1}. This may imply that the hydrocarbon chains are predominantly packed in the hexagonal and orthorhombically packed OL manner, respectively. The spectra of a series of 1:3 diglycerides in the α_L-polymorphic forms are shown in Figure 15.

Information about the chain length can be deduced from the bands in the 8.0 μ (1250 cm^{-1}) region of the spectra. The number of bands observed is equal to half the number of carbon atoms in the chain, $\frac{1}{2}n$ for the "even carbon" diglycerides.

Monoglycerides

Monoglycerides can be of two main types, depending on where the chain is attached to the glycerol residue, i.e. 1-monoglyceride or 2-monoglyceride. (The 1-monoglycerides are optically active but 2-monoglycerides are not.) The spectra of the monoglycerides in the liquid state show the bands to be broad and tending to smear into each other.

A means of differentiating the two types of monoglycerides follows from the fact that different thermal treatment of the 1-monoglycerides gives rise to a number of different spectra, corresponding to the different polymorphic forms in which the 1-monglycerides can occur. The 2-monoglycerides exist in only one polymorphic form.

The spectrum of the crystalline form of a 2-monoglyceride is quite distinct from that of the 1-monoglyceride; this simplifies characterization of the two types. The practical use of the spectra obtained from the crystalline form of 1-monoglycerides has been demonstrated by Kuhrt et al. (66) who showed that monoglycerides were formed to an appreciable extent when monoglyceride-free fat was used to make bread.

Cholesterol Esters

The IR spectra of a number of pure cholesterol esters of long chain fatty acids have been reported (67). The spectral data enables differentiation to be made between the saturated and the unsaturated cholesteryl esters and also to determine the nature of the fatty acids present. All the solution and solid state spectra of cholesterol and its esters show two bands at 11.9 μ and 12.5 μ (840 and 800 cm^{-1}) due to the C–H bending vibration adjacent to the 5,6 double bond of the cholesterol molecule and a very weak band at 6.0 μ (1667 cm^{-1}) assigned to the stretching vibration of the double bond. Strong absorption bands at 3.4 μ (2941 cm^{-1}), 6.8 μ (1470 cm^{-1}) and 7.2 μ (1370 cm^{-1}) are due, respectively, to the CH groups of the methylene and methyl groups of the cholesteryl and fatty acid radicals.

Cholesterol itself shows a band at 2.7 μ (3600 cm^{-1}) and a broader band at 3.0 μ (3330 cm^{-1}) assigned to the O·H stretching vibration of the free and bound hydroxyl group. A strong band at 9.5 μ (1050 cm^{-1}) is assigned to the C–O vibrations of the hydroxyl group. With the esters the hydroxyl absorptions near 3330 cm^{-1} and 1050 cm^{-1} disappear and a strong sharp band at 5.8 μ (1730 cm^{-1}) due to the ester group occurs. The peaks at 8.0 μ (1250 cm^{-1}) and 8.5 μ (1175 cm^{-1}) are associated with the C–O vibrations of the ester linkage.

The solution spectra of the saturated cholesteryl esters are almost identical. In the solid state, however, the spectra of the saturated esters show an increase in the intensity and sharpness of bands at 6.9 μ (1430 cm^{-1}), 8.5 μ (1175 cm^{-1}) and 13.7 μ (725 cm^{-1}). Also a series of regularly spaced bands occur between 7.5 μ (1330 cm^{-1}) and 8.4 μ (1185 cm^{-1}). The number of bands is proportional to the chain length of the compound. Some weaker bands also occur in this region in the spectra of the laurate.

Only one band occurs at 722 cm^{-1} for solid cholesteryl laurate whereas there is a doublet in this region for the higher homologues. This may be an indication of a difference in the chain packing. Spectral changes with temp show that the onset of the liquid crystalline state of these esters takes place in an analogous way to the phospholipids (to be described later).

The unsaturated esters are distinguished from the saturated compounds by the presence of a small peak at 8.8 μ (1135 cm^{-1}) while several weak bands in the 9.0 μ (1110 cm^{-1}) region can be used to differentiate the unsaturated esters from each other. Cholesteryl oleate has a small band at 8.9 μ (1120 cm^{-1}) and an inflection point at 9.1 μ (1105 cm^{-1}), the linolenate has an inflection point at 8.9 μ (1120 cm^{-1}) and a definite peak at 1105 cm^{-1}. The linoleate shows only a shoulder at each of these two wavelengths. All three compounds have a peak at 8.8 μ (1136 cm^{-1}) which increases in intensity with unsaturation and a peak at 9.2 μ (1085 cm^{-1}) which decreases in intensity with increasing unsaturation. The solid state spectra are useful for distinguishing the various homologues. Near IR spectra are not particularly helpful in distinguishing the various esters.

Steroids

Steroids have been extensively studied by IR spectral measurements (68,69). The spectra of more than 3,000 steroids between 7.4 μ (1350 cm^{-1}) and 15.4 μ (650 cm^{-1}) have been investigated. The specificity of this region is illustrated for four stereoisomeric hydroxy-ketones. With such large molecules assignment of bands is difficult if not impossible. The concept of zone patterns has again been applied to spectra obtained on an absolute intensity basis of molecular extinction coefficients. A characteristic zone pattern is set up for each functional group consisting of a Lorentz curve form and a half band width of 1429 μ (7 cm^{-1}). By analysing many spectra the position of certain characteristic groups can be determined (70). These individual bands correspond in position to the bands in the pattern and the height of the curve is taken from the extinction coefficient of the absorption peak of the simple monosubstituted steroid.

Phospholipids

The IR spectra of a number of phospholipids have been published but there are many unsatisfactory gaps and discrepancies in the data. Some of the discrepancies may arise from the fact that the materials under examination were impure, or that acyl migration had occurred during the preparation. In other cases the occurrence of polymorphism has confused the situation so that different spectra have been reported for the same material. As yet, no detailed analysis has been published on the effect of crystallinity on their IR spectra. We discuss first the spectra of the main phospholipid types obtained with the materials in solution and then discuss the more informative but more confusing spectra of the solid state material.

We can make the following observations about the similarities and differences we expect in the spectra of the main classes of phospholipids. All the spectra of the cephalins, lecithins, plasmalogens and the main glycerol derivatives will show bands arising from the glyceryl residue. If they are long chain derivatives, bands arising from the vibrations of the polymethylene chain will also occur. The remaining common feature is the presence of a phosphate group.

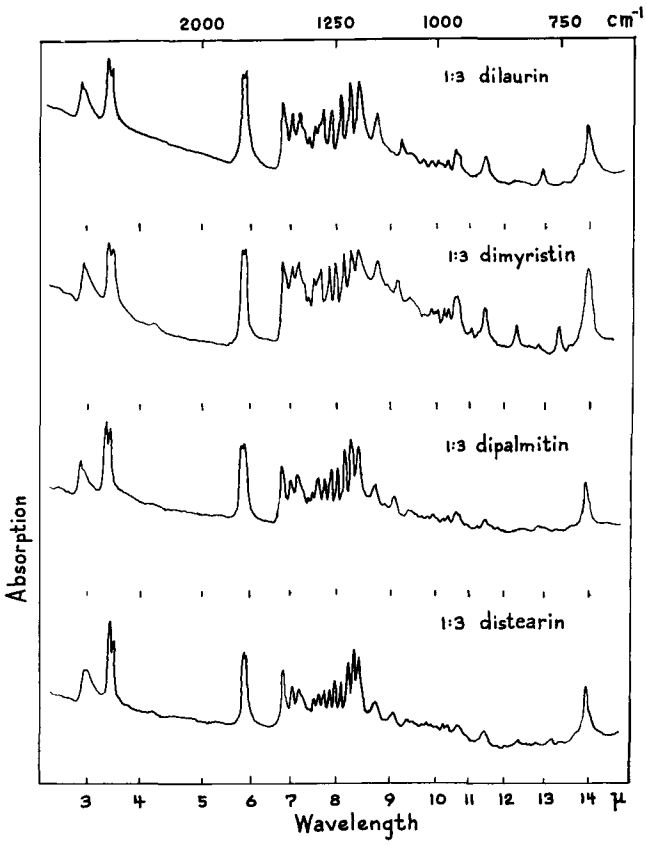


FIG. 15. IR spectra of the β_L-polymorphic forms of some 1:3-diglycerides.

Vibrations of this group usually occur as follows, *a*) P = O stretching, vibration absorption at 7.4 μ (1350 cm^{-1}) to 8.0 μ (1250 cm^{-1}) (v.s.) sometimes a doublet and hydrogen bonding can shift the band to lower frequencies with an increase in intensity, *b*) POC vibration, absorption about 9.5 μ (1050 cm^{-1}) to 10.2 μ (980 cm^{-1}) (v.s.), usually two bands are observed, *c*) POH vibration, absorption in the region 3.7 μ (2700 cm^{-1}) to 3.9 μ (2560 cm^{-1}).

To summarise the expected major difference between the phospholipid classes we note that the phosphatidyl ethanolamines and serines (cephalins) contain an NH_2 or $\overset{+}{N}H_3$ grouping, the lecithins contain an $\overset{+}{N}(CH_3)_3$ grouping, the sphingolipids contain an amide grouping and the plasmalogens do not contain an ester (C = O) grouping. These differences themselves are sufficient using the solution spectra to distinguish these main classes. The region from 5.9 μ (1670 cm^{-1}) to 7.1 μ (1390 cm^{-1}) is obscured by CS_2 absorption. The ester band (C = O) near 5.7 μ (1740 cm^{-1}) to 5.8 μ (1720 cm^{-1}) occurs in the spectra of the cephalins and lecithins but not with the sphingolipids. The sphingolipids have bands at 6.0 μ (1640 cm^{-1}) and 6.4 μ (1540 cm^{-1}). Also bands occur at 10.3 μ (970 cm^{-1}) in the spectra of the lecithin and sphingomyelin but not with the cephalin. [Unesterified cholesterol has essentially no absorption near 5.8 μ (1720 cm^{-1}).] The presence of a band at about 970 cm^{-1} in saturated and unsaturated compounds interferes with measurements of the *trans* unsaturated content. Differences have been observed in the region of the P = O stretching frequency between the phosphatidyl ethanolamines [strong band 8.1 μ (1227 cm^{-1})] and the phosphatidyl serines 8.1 μ (1220 cm^{-1}) to 8.4 μ (1180 cm^{-1}).

Such differences have been used to analyse total serum lipid extracts (71). Simultaneous equations

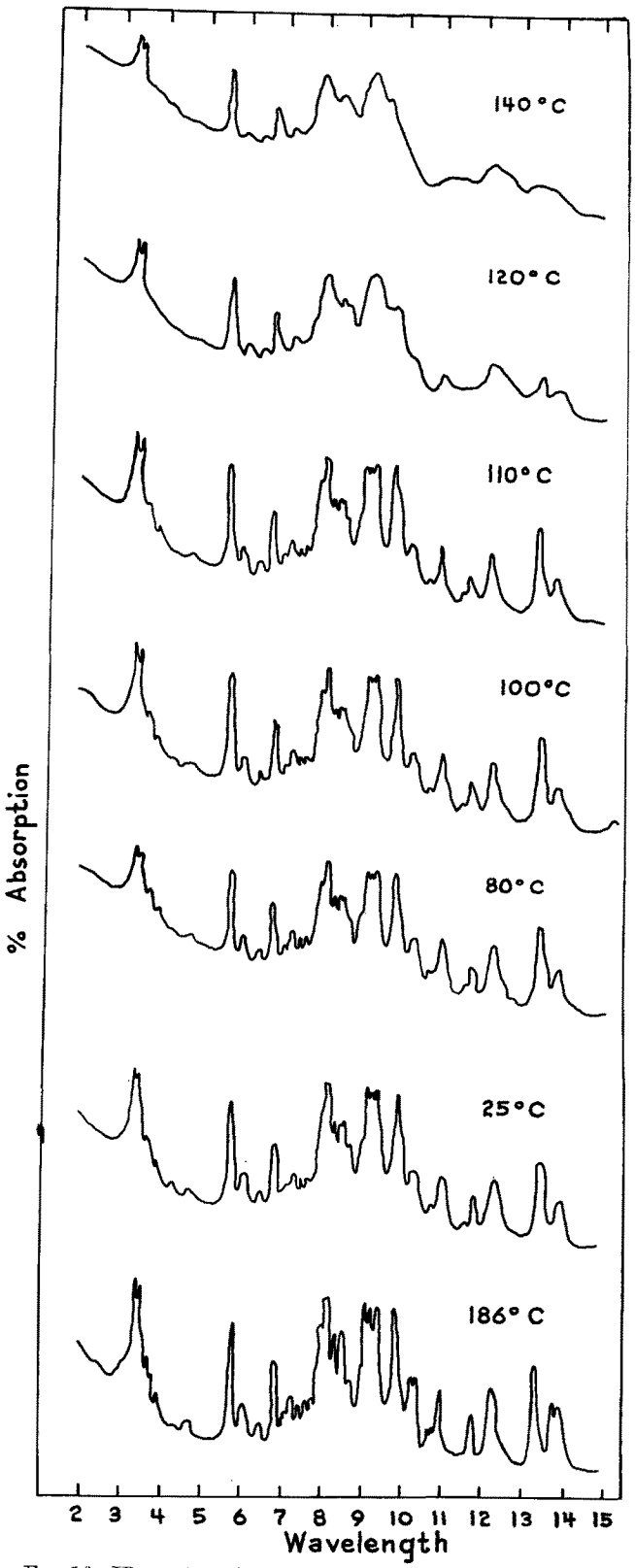


FIG. 16. IR spectra of DL-α-dipalmitoyl phosphatidylethanolamine at different temps.

can be set up and solved for the concns. By making various assumptions and approximations the equations are further reduced so that by intensity measurement at three frequencies the concn of esterified fatty acids, cholesterol and residual phosphatides can be determined. It has been conjectured that such analyses on a routine basis could be very valuable in clinical laboratories.

By using chromatography and IR spectroscopy serum phospholipids have been analysed (72). The extracted lipids are separated into five fractions by successive elution from a silicic acid-celite column with methylene chloride, acetone, 35% methanol, 65% methylene chloride (two fractions) and methanol. The phospholipids are contained in the last three fractions. Using reference compounds the amts of cephalin, lecithin and sphingomyelin have been determined.

A method has also been developed for estimating cholesterol esters and triglycerides simultaneously in serum lipids (73). This involves separating the phospholipids from the total lipid mixture by a simple batch absorption on silicic acid. Cholesterol esters and triglycerides are the main constituents in the nonadsorbed lipid and measurements are made on this mixture. The analysis of the two components is based on the fact that the ester carbonyl absorption frequency for cholesteryl esters is different from that of triglycerides by about 714 μ (14 cm^{-1}). For this analysis it is necessary to have the resolution and precision of a grating spectrophotometer. Pure triolein and pure cholesteryloleate are used as standards and the absorption coefficients obtained at the two selected frequencies. The oleate absorbs at 5.8 μ (1730 cm^{-1}) and the triolein at 5.7 μ (1745 cm^{-1}). There are two requirements for this method, *a*) the silicic acid adsorbtion must separate out all the phospholipids but none of the components to be measured, *b*) the contribution of other nonadsorbed lipids must be small enough to be manageable. The nonadsorbed lipid material contains unesterified cholesterol and unesterified fatty acids. The cholesterol molecule has no carbonyl group while the absorption arising from the free fatty acids can be less than 5%. The acetone solvent must be completely removed from the lipid.

Spectral studies have been made with sphingomyelins (74). The presence of a band at 10.3 μ (970 cm^{-1}) was associated with the existence of a *trans* configuration of the double bond in sphingomyelin, N-lignocerylsphingosine and cerebroside. After hydrogenation the band disappears with the latter two compounds and is diminished with the sphingomyelin which exhibits another band in this region. It has been suggested that lecithin may be quantitatively determined when present in mixtures with sphingomyelin.

The IR spectra of a number of natural phospholipids have been examined by Rouser et al. (75). They show the spectra of a brain lecithin, phosphatidyl ethanolamine and sphingomyelin, a synthetic saturated dimyristoyl phosphatidyl ethanolamine and a partially hydrogenated phosphatidyl ethanolamine. These workers pointed out that the spectra of the natural phosphatidyl ethanolamine is quite different from that of the synthetic material and suggested that hydrogenation of the natural material may be a useful technique to adopt in order to aid identification. They also commented that their spectra of the dimyristoyl phosphatidyl ethanolamine was different from that of other workers. It was also observed with the natural phospholipids that differences in chain length, unsaturation and even the presence of an α,β-unsaturated ether link did not cause appreciable changes in the spectra. The spectra of acidic lipids were also studied and it was noted that the free acid and the salt form give different spectra. It has been stated that IR spectroscopy is useful for characterising the hydrolysis products of lipids. The sample is hydrolysed and separated into water-soluble

and organic solvent-soluble fractions and the weight of these determined. The solutions are then chromatographed and their spectra obtained. If possible this is compared with that of a model substance.

The spectra of a number of synthetic phospholipids have also been published (76). The spectra of two isomeric lecithins containing one stearic acid and one oleic acid chain in different positions were shown to be completely identical and much less differentiated than that of a fully saturated lecithin. This behaviour is also observed with phosphatidyl ethanolamines, and also with the serine-containing glycerophosphatides and lysolecithins.

The reason for this contrast between the spectra of the natural, the unsaturated phospholipids and the saturated phospholipids became clear recently as a result of an investigation of the spectral variations observed with temp (77). DL-α-dipalmitoyl phosphatidyl ethanolamine was crystallised from chloroform and made into a KBr disc. The spectrum at room temp (Fig. 16) corresponds to that of an A′ form. At low temp (−186C) the spectrum shows a great deal of fine structure, e.g. the band near 13.9 μ (720 cm^{-1}) associated with the CH_2 rocking mode is split into a doublet. At room temp some of the fine structure has disappeared and now only a single band occurs at 720 cm^{-1}. As the temp increases up to about 100 to 120C all the remaining fine structure disappears and the spectrum resembles that of a liquid rather than a solid, although the "true" melting point of the lipid is some 195C. This is analogous to the behaviour observed with sodium soaps and the interpretation is the same, i.e. as the temp increases the hydrocarbon moiety begins to flex and twist until melting of the chain occurs. The ionic end groups retain the crystalline character of the material until the true melting point is reached. This interpretation is consistent with the decrease observed in the X-ray long spacings and the fact that the short spacings form diffuse halves at ~4.5Å when the temp is above about 100C.

The variation of the spectra with temp may be interpreted on the basis that the hydrocarbon chains are in the orthorhombic $O_\perp$ type packing arrangement at the lowest temp while at higher temp the packing approaches that of the hexagonal type and finally becomes liquid-like.

This observation has a number of implications because the phospholipids containing both saturated and unsaturated chains will have the corresponding transition temp at a lower temp and it is clear that with a phospholipid containing a mixture of saturated and unsaturated fatty acids the hydrocarbon chains can be in an essentially liquid condition at room temp despite the fact that the lipid has a 'true' melting point of some 200C. This is the reason why the spectra obtained at room temp of a variety of natural lipids of this type show no significant difference between related isomers. (This liquidity of the hydrocarbon chains well below the melting point of the lipid is probably the reason why synthetic membranes prepared from natural phospholipids are quite different under the electron microscope from those prepared with phospholipids containing saturated fatty acids). This liquid character of the chains in the natural phospholipids is clearly of considerable importance for the function of these lipids in the biological system and when water is present will determine the particular type of phase which can exist at any particular temp and be in turn related to permeability processes.

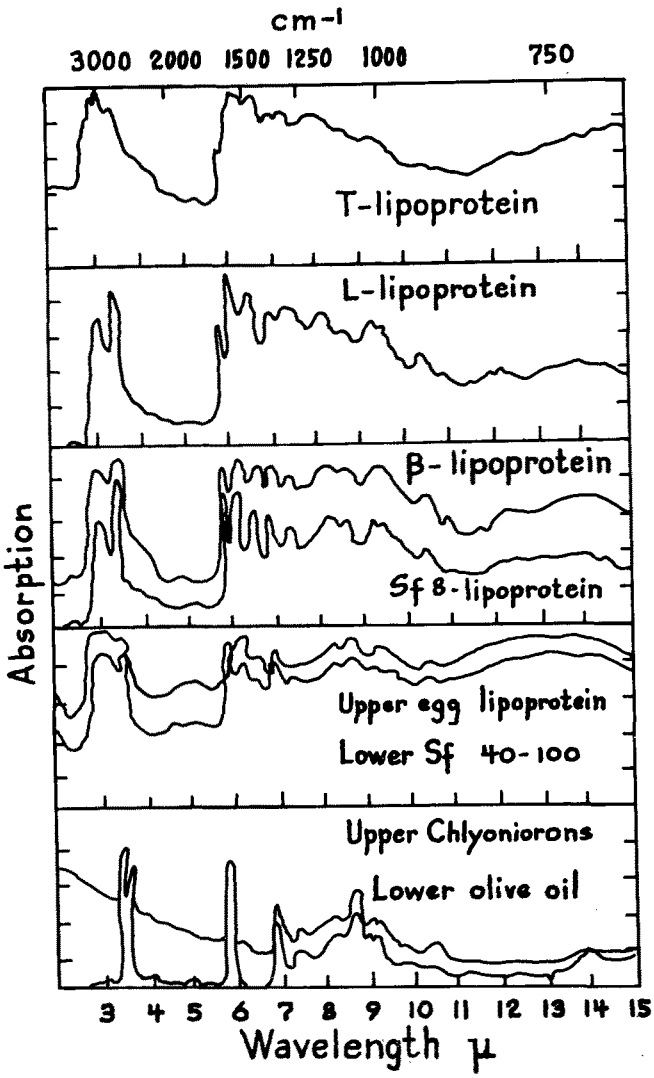


FIG. 17. IR spectra of lipoproteins as dried films. (Freeman, N. K. *N. Y. Acad. Sci. 69*, 131, 1957.)

With a phospholipid containing saturated and unsaturated fatty acids but where there is a *trans* grouping present rather than a *cis* double bond in the 9–10 position we should expect the transition temp for melting of the hydrocarbon chains to be a little higher than the *cis* but not quite as high as with the corresponding fully saturated phospholipid. The presence of linoleic or linolenic acids in the phospholipids will presumably lower the transition temperature.

It is also clear that, in order to determine whether differences occur between related isomers containing unsaturated and saturated fatty acid chains, it is necessary to cool the phospholipid below the transition temp when the hydrocarbon chains crystallise.

This has been done with a 1-stearoyl-2-oleyl lecithin (78). At room temp the spectrum of the material in a KBr disc shows that the hydrocarbon chains are in a liquid-like condition. On cooling the disc slowly down to liquid nitrogen temp the spectrum sharpens and shows considerable fine structure. On raising the temp to room temp some of this fine structure disappears. However, it does not revert to the original spectrum until a temp between 40 to 50C is reached. Further work is at present being carried out to ascertain whether the original liquid-like state of the phospholipid is due to the transition temp being exceeded during the preparation of the KBr disc. (It is possible for a material to crystallise directly into

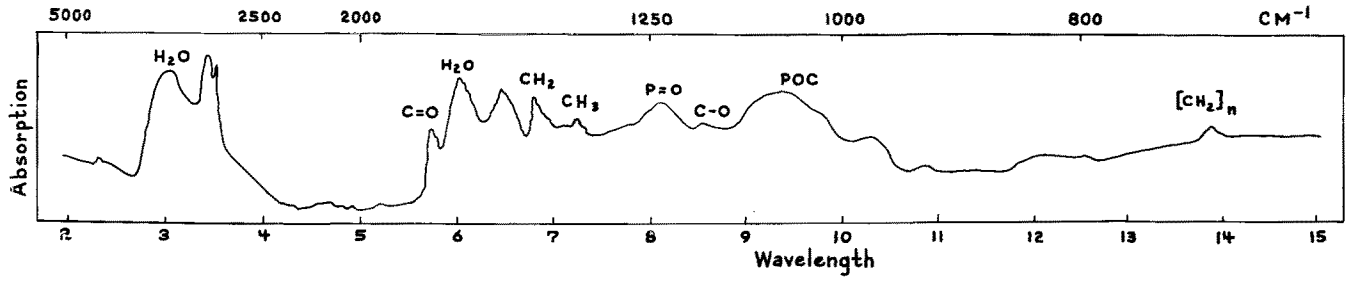


FIG. 18. IR spectrum of myelin sheath of pig optic nerve. Chapman, D. "The Structure of Lipids," Merhuen, 1965.

the liquid crystalline form.) It will be remembered that the closely related isomer 1-oleodistearin and 2-oledistearin give identical spectra when examined as liquids but marked differences appear when they are in the crystalline state so that it may be possible with low temp spectra to obtain differences with phospholipid isomers. (In view of the occurrence in nature of the unsaturated acid in the 2-position of glycerol it is of some importance to determine whether this transition temp differs from that of the isomer with the unsaturated acid in the 1-position.) The literature concerning the IR spectra of the saturated phospholipids examined in the solid state shows considerable confusion (75,79). This has been previously commented upon without completely resolving the situation. There are differences in the reported spectra of the same compound, e.g. of L-dimyristoyl-α-cephalin, and unexplained differences between the spectra of homologues, i.e. large unexplained spectral differences exist between, say, the dipalmitoyl cephalin compared with the distearoyl and dimyristoyl compounds. Some of these differences are attributed to the occurrence of two polymorphic forms for these materials. When DL dipalmitoyl-α-cephalin is crystallised in two different ways from a chloroform-methanol mixture and from ethanol two different spectra, referred to as A and A′ spectra, occur. The major differences between the A and A′ spectra are:

a) The ester carbonyl band in the A-spectrum is a sharp band at 5.8 μ (1739 cm^{-1}) while in the A′ spectrum there is an asymmetric absorption band at 5.9 μ (1725 cm^{-1}).

b) A band occurs at 6.4 μ (1563 cm^{-1}) for the A-spectrum but at 6:5 μ (1550 cm^{-1}) for the A′-spectrum.

c) Many differences occur between 9.5 μ (1050 cm^{-1}) and 14.9 μ (670 cm^{-1}). In particular a weak band occurs at 13.4 μ (745 cm^{-1}) with the A spectrum but there is a strong absorption band at this frequency with the A′ spectrum.

We have confirmed that this arises from polymorphism by X-ray study. Only one polymorphic form had previously been reported to occur at room temp with these phospholipids but more recent studies now show the existence of other forms. The chain length of the fatty acids present in a saturated phospholipid can be determined by heating it to the top or true melting point and then cooling to room temp. The pattern of bands in the 8.0 μ (1250 cm^{-1}) region of the spectra enable one to determine this easily. A more complete study of the polymorphic behaviour of phospholipids is at present in progress.

It seems probable that just as with the glycerides the IR spectra of the phospholipids in the solid state will reveal *a*) the type of phospholipid present, *b*) the polymorphic form in which it occurs as well as information on the hydrocarbon chain packing, *c*) the chain length and any unsaturation which is present, such as the *cis* or *trans* groupings, *d*) in some cases the positions of the fatty acids in the phospholipid, i.e. whether they are in the 1,2 or 1,3 positions on the glycerol moiety, *e*) whether the material is a DL mixture or is that of one of the optical isomers.

It has been suggested that absorption in the 4.0 μ (2500 cm^{-1}) to 3.7 μ (2700 cm^{-1}) region can be associated with P–OH stretching vibrations of the phosphatidyl ethanolamines when in the solid state (80). The small band observed near 4.8 μ (2100 cm^{-1}) to 4.3 μ (2300 cm^{-1}) may also be related to vibrations of the P–OH group but it should be pointed out that a weak band near 4.8 μ (2100 cm^{-1}) is often observed where NH_3^+ groupings occur in a molecule. It was concluded that this is an indication that these molecules do not exist in a zwitter-ion form in contrast to the lecithins. The frequencies of absorption of the NH_2 stretching and deformation vibrations of the cephalins were considered to be additional support for this conclusion. The suggested coexistence of both OH and NH_2 groups in the same molecule might then be related to the fact that the OH group could be strongly hydrogen bonded perhaps to one of the carbonyl groups. However, this conclusion is by no means certain and it could be argued to the contrary that the evidence favours a zwitter ion structure and the presence of an NH_3 grouping. Single crystal X-ray studies will probably be necessary to confirm these speculations. The high melting points of the phospholipids suggest that ionic groups occur linking molecule to molecule throughout the lattice.

Lipoproteins

The absorption of IR radiation by water is very strong and so lipoproteins have usually been examined in the dry or nearly dry condition. One method used is to evaporate lipoprotein solutions on a silver chloride plate and to examine the spectra of the dried films. Another method is to freeze-dry the intact lipoprotein with potassium bromide and press the powdered mixture into a pellet.

The spectra of a number of lipoproteins obtained from ultra-centrifugally isolated serum samples have been compared with model substances (81). The molecular species were characterized by their rate of flotation in a given medium and under a specified field of centrifugal force. The spectra of some of these lipoproteins (Fig. 17) are shown corresponding to increasing flotation rates. The order is the order of increasing lipid content. The various prominent bands have been assigned as follows — 5.8 μ (1730 cm^{-1}) ester carbonyl, arising from glycerides, cholesteryl esters, and some phospholipids including lecithin, 3.4 μ (2940 cm^{-1}) to 3.5 μ (2860 cm^{-1}) — the absorption bands of the CH groups from fatty acid chains and cholesterol. The principal protein bands are at 3.0 μ (3330 cm^{-1}) — the NH stretch 6.1 μ (1640 cm^{-1}) — C = O stretch — and the band at 6.5 μ (1540 cm^{-1}) assigned to monosubstituted amides. The

L, T and S_F6 lipoproteins are normal serum constituents. A comparison between the spectra of lipoproteins obtained in different ways is possible. β_1-Lipoprotein and S_F6 lipoprotein spectra are very similar.

Fractionation of the lipoprotein with solvents separates the protein and lipids. The spectra of some of the lipoprotein fragments from egg lipoprotein after successive extraction are shown in Figure 17. These have been compared with those of some reference materials and it has been shown that fat predominates in the extracted mixture. Acetone soluble material from S_F6 lipoprotein gives cholesterol while the acetone insoluble material from human serum contains mainly lecithin.

An analytical method has been developed for semiquantitative determination of lipoprotein composition in terms of the major constituents by the use of intensity measurements at appropriate wavelengths. After evaporation of solvent the lipid extracts are dissolved in measured volumes of CS_2 and the spectra obtained. The concns of the extracted lipids can then be estimated. The acetone-soluble lipids are calculated as cholesteryl esters or as fat. If a distinct band appears at 9.2 μ (1050 cm^{-1}) unesterified cholesterol may be estimated. The acetone-insoluble lipids are calculated as lecithin. The lecithin-protein ratio is obtained from the relative intensities of the absorption bands at 5.8 μ (1724 cm^{-1}) and 6.5 μ (1540 cm^{-1}). (The presence of sphingomyelin is neglected.) Various improvements on the method have been suggested including the consideration of minor constituents but fairly good agreement is obtained. More recently Freeman and co-workers have used silicic acid-Celite columns, taken the eluates from the columns, evaporated them to dryness and redissolved in CS_2. The three fractions from the column are identified and estimated by IR spectroscopy. Fraction 1 contains the cholesteryl esters and bands at 5.8 μ (1724 cm^{-1}) or 8.5 μ (1170 cm^{-1}) are used. Fraction II contains the glycerides, unesterified fatty acids and unesterified cholesterol and bands at 5.7 μ (1740 cm^{-1}), 5.8 μ (1710 cm^{-1}) and 9.5 μ (1050 cm^{-1}) are used in a multicomponent analysis. Fraction III contains phospholipids and a band at 9.3 μ (1070 cm^{-1}) is used for quantitative estimation. Serum phospholipids and milk phospholipids have also been examined in this way. In the latter study each fraction was analysed by spectra from KBr discs and compared with pure compounds.

An IR spectroscopic study of myelin sheath is at present in progress and may produce useful results (82). A typical spectrum is shown in Figure 18. The spectrum shows the strong absorption due to the water present but also bands which may be tentatively related to the phospholipid material and the assignments are indicated in the figure. It appears from the spectrum that the hydrocarbon chains are in a liquid-like condition. This is consistent with the observed X-ray short spacing at about 4.5Å. This does not necessarily preclude some order among the chains. A band near 6.5 μ (1540 cm^{-1}) may perhaps be related to protein material present but additional studies are needed to confirm this. Further studies using D_2O and also studies at different temps should provide additional useful information. There is also the possibility that polarized IR radiation may be useful in helping to confirm the opinions reached from optical, X-ray and electron diffraction studies on the spatial arrangements of the phospholipids.

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