Applications of Infrared Absorption Spectroscopy in the Analysis of Lipids[1]

NORMAN K. FREEMAN, Donner Laboratory of Medical Physics and Biophysics, Lawrence Radiation Laboratory, University of California, Berkeley, California 94720

Abstract

The uses of infrared spectroscopy in lipid chemistry are discussed, with main emphasis on quantitative analytical methods. In reviewing the infrared technique from an overall perspective, attention is also given to instrumental developments, sampling techniques and qualitative applications. Some features are brought out in the context of examples cited. These include some significant established methods as well as recent developments, such as the use of integrated band intensities and the involvement of computers and computer techniques in infrared analysis. Current trends and future directions are indicated, and some unrealized potentialities are suggested.

Introduction

INFRARED SPECTROSCOPY is no longer a new technique. It is indeed the oldest of the spectroscopies, if we take it to have begun with Coblentz in the early years of the present century. However another possible point of origin can be designated as the time of reduction to practice. This can be said to have taken place with the advent of commercial infrared spectrometers at the end of World War II, predating most of the methods discussed in this symposium. It seems proper, then, to think of infrared spectroscopy as a mature field, one which is still growing, but at a much slower rate than before. New developments and new applications are currently appearing with less frequency than they did during the 10- to 15-year postwar period of extremely rapid growth.

Looking back on those early years, it is well to bear in mind that many of the other methods in current use today had not yet become competitive. Infrared appeared on the scene as a tool of outstanding promise in a tremendously wide variety of analyses and chemical research areas. Its virtues then are its virtues today. Instrumentation was (or soon became) reliable, simple to operate, and comparatively low in cost. Samples could be in solid, liquid, gaseous or solution form, and the amount of material required was small. The method was nondestructive, allowing samples to be recovered for further use. Fundamentally however its most important attribute was the great wealth and variety of information inherent in the infrared spectrum. In contrast to the melting point or refractive index, which are single-valued properties of a substance, the infrared spectrum is a multivalued property. To the extent that the spectral information can be extracted or interpreted, it may provide a multiplicity of descriptive items characterizing the substance. This represents a kind of supercharged qualitative analysis, described by Thompson as "structural diagnosis" (1). In addition to its qualitative applications, infrared presented a host of new opportunities for quantitative analysis. As a result, infrared spectroscopy developed rapidly and was enthusiastically adopted in many diverse branches of chemistry, usually with substantial success.

As the infrared field was developing in a healthy and fruitful way during the decade between 1950 and 1960, other techniques were either being introduced or brought to the stage of general utility, i.e., they were adopted on a fairly wide scale for analytical purposes. Mass spectrometry and nuclear magnetic resonance were experiencing gradual growth, possibly retarded somewhat by the relatively high cost of the equipment. Gas chromatography on the other hand, with its relatively low cost and extreme versatility, proliferated in a spectacular fashion. Other examples might be cited from an extensive list of methods—principally instrumental ones—that have been developed and refined during this period and on up to the present time. The resulting situation is one in which an array of powerful physical techniques is available to the analyst, and he may choose from among them, either singly or in combination, for the attack on a particular problem. For structural investigation the combined approach is to be favored, with each technique contributing as a member of the team. In a recent book (2) (designed for teaching purposes) this concept is developed by a set of illustrative examples. In each example or problem, the infrared, ultraviolet, nuclear magnetic resonance and mass spectra of an unknown compound are presented, together with additional information such as elemental composition. The student is asked to find the relevant information in each spectrum, assemble and correlate it, and by inference deduce the structure. It is stated that this mode of presentation is "closely akin to contemporary research techniques." Indeed, in the field of fatty acid chemistry it may be exemplified by the work of Paschke et al. (3), who used this kind of concerted spectroscopic approach in their study of the structure of dimer acids. In the light of current technological trends, other spectroscopists now propose that this interpretive function be carried out by computers. Thus in a fully automated laboratory virtually at hand, spectral and other data would go directly to a computer, which would be programmed to recognize significant items of information, would compile them, and would arrive at logical conclusions about the structure or identity of the sample. Such a system has been put into practice in Japan, according to a recent report (4).

If, on the other hand, the analytical problem is one of quantitative measurement, it is more likely to require only a single method rather than a combination. A decision has to be made as to which method or methods can be applied in the given situation and, if there is a choice, which method best meets the analytical criteria of sensitivity, accuracy, specificity, operational simplicity, etc. It is in this area that techniques become competitive rather than complementary to one another. And it is here that infrared spectroscopy has lost some of its early preeminence. It still has its basic capabilities, and they are greatly improved, but now they may have to

[1] Presented at the AOCS-AACC Joint Meeting, Washington, D.C., April, 1968.

be weighed against other available methods. In that perspective, it is our concern here to indicate some of the analytical applications of infrared absorption, without attempting to make comparative judgments in relation to other methods.

Instrumentation

Before proceeding to specific analyses in the lipid field, a very brief review of the development of instrumentation and technique may be in order. The great profusion of infrared spectrophotometers available today are for the most part descendants of the simple, single-beam, sodium chloride prism instrument which was manually operated point-by-point, with visual observation of galvanometer deflections. Such an instrument was better adapted to quantitative analysis than to obtaining full spectral curves. In fact gas analysis was one of the first broad-scale applications of infrared absorption. The first commercial double-beam spectrometer soon appeared (1947); and its capability of directly recording transmittance curves over the whole spectral range in a few minutes set the pattern which still prevails today. This type of instrument was more attractive to the chemist who was not spectroscopically oriented and it facilitated the growth of large libraries of reference spectra. It tended to favor the development of qualitative applications. For a number of years the double-beam automatic recording prism spectrometer was prevalent, with two sub-classifications: low-cost, simple-to-operate types of limited flexibility, and more expensive, versatile research instruments with variable scanning speeds, slit programs, etc. In some instances double monochromators were used to improve resolving power. The substitution of diffraction gratings for prisms was the next significant advance, and for the past several years the majority of new instruments have used gratings. This represents further improvement in wavelength resolution, and also an extension of the readily available wavelength range. It is much easier to go beyond the sodium chloride limit of 16 μm with a grating than it is to substitute a different kind of prism. Furthermore there are no known materials of sufficient transparency beyond about 50 μm to use as prisms, while one commercial spectrometer equipped with gratings operates to 300 μm. One of the difficulties which retarded the earlier development of grating instruments was the necessity for separating the various spectral orders obtained from the grating. Formerly accomplished by means of a foreprism, order separation is now more frequently done with filters. The development of the technology for making infrared filters was probably the key factor in initiating the trend toward the general use of grating spectrometers.

Some other recent developments in instrumentation include interferometric spectrometers and rapid-scan spectrometers. The former (5) is based on a Michelson interferometer; in operation it yields an interferogram, which must be translated into a conventional spectral absorption curve by Fourier analysis. This involves the use of a computer. An interferometer can be used in any part of the infrared spectrum, but its primary advantage over dispersive spectrometers is its efficient use of radiation. Consequently its applications have been mainly in the far infrared and in the study of emission spectra at relatively low temperatures, both of which are low energy situations. Repetitive scans with signal accumulation are commonly employed as a means of averaging out noise. Coleman and Low (6) have obtained emission spectra of microgram quantities of pesticides with this type of spectrometer. Holman (7) has presented some far infrared spectra of methyl esters obtained with a grating instrument (to 300 μm), but to the author's knowledge no applications of far infrared spectra in the field of fats and oils have been reported.

Rapid-scan infrared spectrophotometers can be classified into two types: very fast instruments capable of scanning a spectrum in a few microseconds, relatively fast instruments with scan times of a few seconds (e.g., 5 sec for Beckman Instruments Model 102). The former are not commercially available, but have been especially built in a few laboratories for the study of fast reactions, transient species, etc. (8). The second category finds its principal application in obtaining spectra of gas chromatograph effluents.

Technique has to do basically with sample handling or the manner in which a substance is presented to the spectrometer for obtaining its spectrum, and with auxiliary devices which permit special conditions of sample handling. It has already been mentioned that spectra can be obtained of samples in gas, liquid or solid state, or in solution. With the standard sizes of cells, and the usual optical geometry of most spectrometers, the amount of sample required is of the order of a few milligrams. One of the earliest accessories brought into use was the beam condenser, for the purpose of reducing sample size to the range of a few micrograms. A beam condenser may consist of either a lens system, as in the simplest type (9), or a reflecting optical system such as the so-called infrared microscope (10). Another important sampling development of the early 1950's was the pressed pellet technique (11), in which a solid sample is incorporated into an alkali halide pellet (disk or wafer) under high pressure. This technique has some definite advantages over earlier methods of preparing solid samples, and it is now in common use. Among its advantages it provides a basis for quantitation, and it facilitates handling very small amounts of material. These factors, together with the use of a beam condenser, are illustrated by a method recently used in our laboratory for the analysis of chylomicron-containing fractions from blood serum (12). Chylomicrons are the large fat-bearing particles which because of their low density can be easily floated by centrifugation. If this isolation is carried out in a properly designed salt gradient, the top layer after centrifugation contains only the very low density lipoproteins (mostly chylomicrons) in aqueous saline suspension. By lyophilizing an aliquot of this, a dry mixture of the chylomicrons in an excess of NaCl is obtained. A pellet 1.5 mm in diameter is pressed from this mixture and placed in the beam condenser for spectral measurement. The ester carbonyl band of the triglycerides is used as a measure of chylomicrons (assuming uniform content of 85% triglycerides). Some calibration curves for this analysis are shown in Fig. 1. Although residual water is present in the NaCl pellet, there is no difficulty in drawing a baseline for the carbonyl band. A virtue of this method is that it requires very little manipulation of the sample that would risk loss or exposure to possible contamination.

Another technique that is not new but continues to find profitable application in the study of crystal

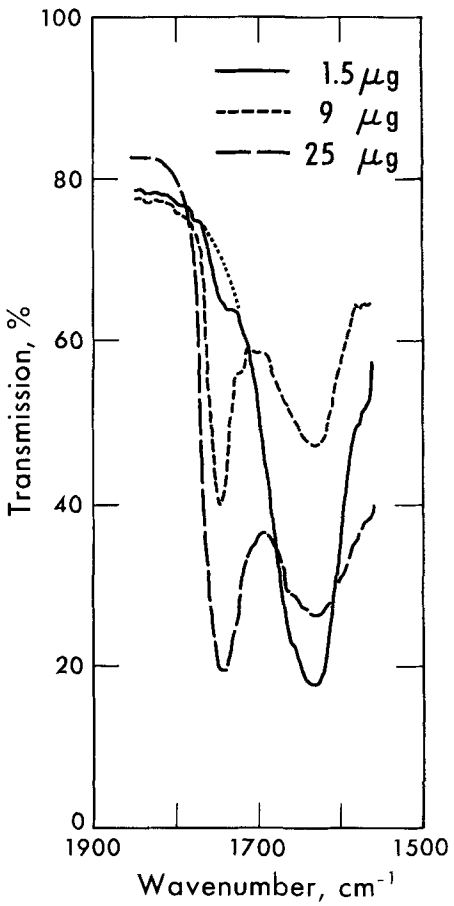

FIG. 1. Infrared absorption bands of triglycerides (1742 cm^{-1}) and residual water (1640 cm^{-1}) in NaCl pellets made from lyophilized chylomicron preparations. The dotted line is a sketched-in background for the solid curve (12).

structure is the use of polarized infrared radiation. Among the early applications of this technique were investigations of bond orientation in polypeptides and fibrous proteins as long ago as 1949 (13). A considerable amount of work of this kind has been done with fatty acids and their derivatives, notably by Susi and co-workers. A recent example (14) is their study of thin crystalline films of methyl stearate and methyl 1-octadecyl sulfonate. Polarized spectra of the latter compound are shown in Fig. 2, illustrating the striking differences that can occur in infrared absorption bands as the direction of polarization is changed. Correlation of these observations with identifiable vibrations and with x-ray data provides valuable information about bond directions and molecular arrangement in the crystal.

Spectral changes as a function of temperature have been studied in relation to polymorphism and phase transitions in fats and fatty acid derivatives. Much of this has been done by Chapman, and the topic is thoroughly reviewed in his recent book, "The Structure of Lipids" (15). Spectra of the polymorphic forms of tristearin are reproduced in Fig. 3 (16). Although the detailed differences in the solid phase spectra are not apparent without close inspection of the curves, the disappearance of many bands on transition to the liquid phase is obvious. A significant observation is the occurrence of singlet or doublet peaks near 720 cm^{-1}, which can be related to the alignment of chains in various crystal forms. Chapman (16) has pointed out that in certain phospholipids the spectra show the characteristics of a liquid at temperatures considerably below the observed melting point. This is interpreted as a partial melting of the hydrocarbon moiety.

A technique which is now widely used for infrared spectroscopic examination of surfaces is known as attenuated total reflectance (ATR). This makes use of an auxiliary optical system in which the key element is an internally reflecting prism of high refractive index. A substance brought into contact with the reflecting surface will act as an absorber of the reflected energy at the same wavelengths at which it absorbs in the normal transmission optical arrangement. Thus the reflectance spectrum resembles very closely the transmittance spectrum. Attenuated total reflectance is a specialized sampling device which is useful for solid surfaces, coatings, etc., but apparently there are no outstanding examples of its use in lipid analysis. However, at the national meeting of the American Chemical Society in April 1968, a paper was listed in the program with the title "Direct Determination of Composition of Foods by the Infrared Attenuated Total Reflectance Technique."

Qualitative Analysis

Qualitative analyses based on infrared spectra can be simple comparison of curves, where an unknown compound may be identified by matching its spectrum to that of a pure reference compound, or a recognition of characteristic absorption bands of functional groups or other structural features (isomeric forms,

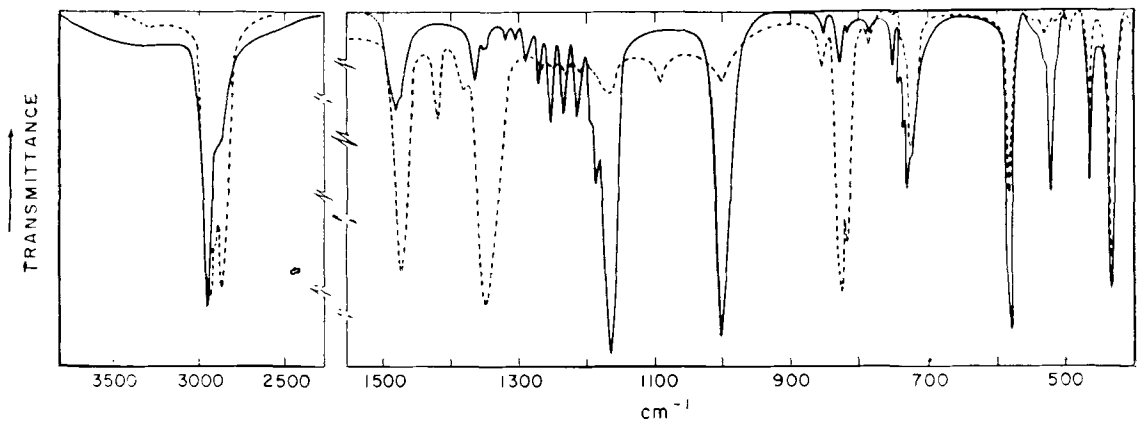

FIG. 2. Infrared absorption spectra of a crystalline film of methyl 1-octadecyl sulfonate, using plane-polarized radiation. Solid line: electric vector parallel to direction of crystal growth. Broken line: electric vector perpendicular to direction of crystal growth (14).

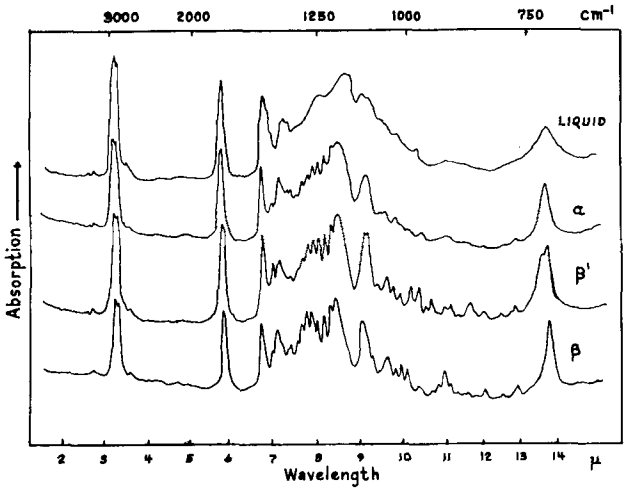

FIG. 3. Infrared absorption spectra of polymorphic forms of tristearin (16).

etc.). Although the first approach, commonly known as the fingerprint technique, is undoubtedly used to some extent in the lipid field, it may fail to distinguish homologues. The spectral features of polymethylene chains tend to persist through all lipid spectra, and the distinguishing features are those of the chemical structures attached to the chains. Consequently we are more concerned with spectral manifestations of those chemical structures. One of the earliest presentations of the infrared spectra of long chain compounds was that of Shreve et al. (17) in 1950. They reported spectra of fatty acids, alcohols, methyl esters and triglycerides, and pointed out the characteristic features of each class. They also indicated the absorptions of *cis*, *trans*, and terminal double bonds. In another publication (18) the same authors were the first to describe the infrared determination of *trans* double bond isomers in the various classes of long chain compounds, based on the absorption band at 10.35 μm. Other studies of the spectra of fatty acids and esters were made by O'Connor et al. (19), and by Sinclair et al. (20,21). The latter authors noted the characteristic band progressions in the 1200–1350 cm^{-1} region of the spectra of crystalline long-chain compounds, and this feature was analyzed in some detail by Jones et al. (22). In more recent papers (23,24) the use of these band progressions for the determination of chain length has been described.

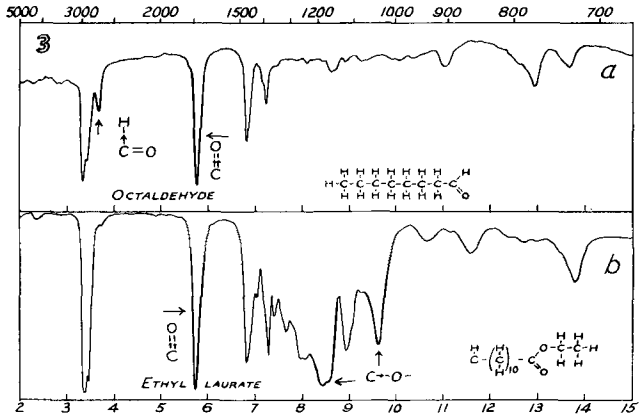

FIG. 5. Infrared absorption spectra of octaldehyde and ethyl laurate (25).

Functional Groups

A few of the basic features of the spectra of long-chain compounds are illustrated in Figs. 4 to 8 (25). They all consist essentially of hydrocarbon chain absorptions (3.4 μm, 6.85 μm, 7.25 μm and 13.85 μm) with superimposed bands of various groups. In addition to those shown, many other important functional groups have characteristic bands, e.g., amines (primary), 3.0 μm (doublet) and 6.1 μm; nitriles, 4.4 μm; ethers, 9.0 μm, etc. Group correlation tables have been given by Colthup (26), and bands useful in fatty acid chemistry were listed in an earlier review by O'Connor (27). For the detection of unusual fatty acids in natural oils, Wolff and Miwa (28) have indicated some characteristic band positions, as in Figs. 9 and 10. The intensities of these bands vary, and in some cases small, or even moderately large amounts may escape detection without prior separation or enrichment.

Chain Branching

In addition to the recognition of functional groups, some information can be obtained about chain branching in fatty acids (29,30). A methyl branch within five carbons of the carboxyl causes observable shifts in two bands at 7.8 μm and 8.1 μm, and only the α-methyl produces a reversal in their relative intensities. Branching near the distal end of the chain can be detected if it gives rise to a terminal ethyl or propyl group. Bands characteristic of these groups are shown in Fig. 11. *Gem*-dimethyl structures, including isopropyl and tertiary butyl groups, cause

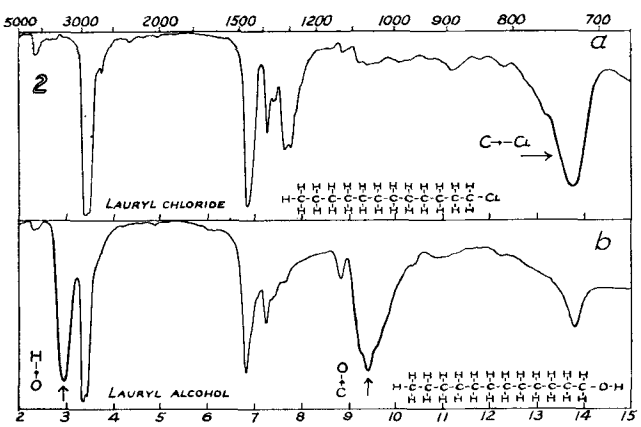

FIG. 4. Infrared absorption spectra of lauryl chloride and lauryl alcohol (25).

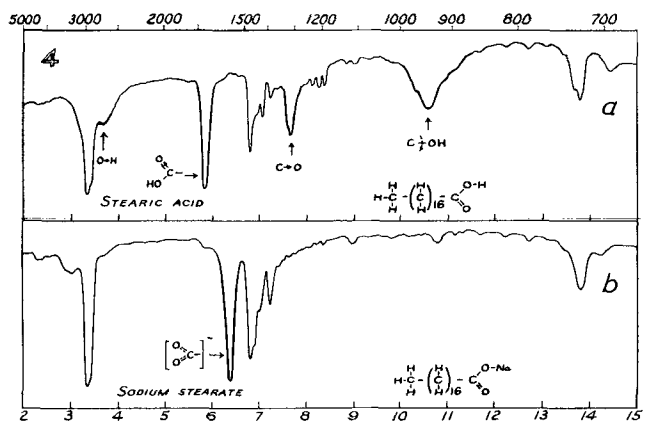

FIG. 6. Infrared absorption spectra of stearic acid and sodium stearate (25).

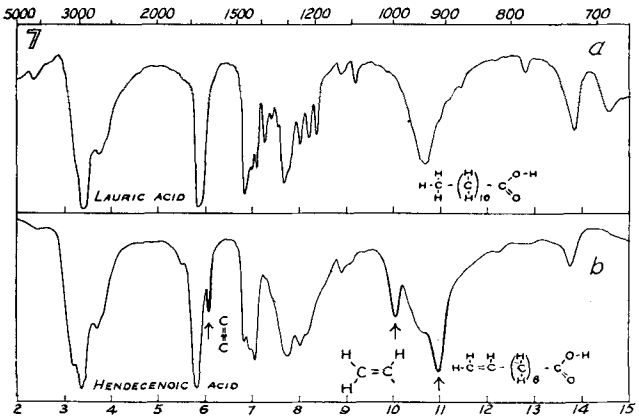

FIG. 7. Infrared absorption spectra of lauric acid and hendecenoic acid (25).

the methyl group absorption at 7.25 μm to split into two components (Fig. 12). The isopropyl group can be further distinguished by an additional band at 8.55 μm.

Chain Length

Features related to chain length in solid fatty compounds have been mentioned above. A simplified method for estimating the average chain length in saturated fatty acid esters has been described (31), based on the ratio of intensities of the CH_2 band at 3.4 μm and the ester band at 5.75 μm. It was established for triglycerides, monoglycerides and methyl esters that the ratio of measured absorbances at these wavelengths bears a linear relationship to the number of carbon atoms in the chain.

Unsaturation

In both hydrocarbon and lipid research, absorptions associated with carbon-carbon double bonds have been extremely useful. In general, the spectral features of various kinds of double bond structures are quite distinctive from one another. The simplest type of unsaturation, which is also the most prevalent in natural fats and oils, is the unconjugated *cis* double bond. Figure 13 (7) shows spectra of methyl esters containing 0, 2, 4 and 6 double bonds of this type. The peaks related to double bonds are at 3.3 μm (= C–H stretching), 6.1 μm (C = C stretching), 7.2 μm (= C–H planar bending), and 14.2 μm (= C–H nonplanar bending). Not shown in this figure is a peak in the near infrared (2.15 μm)

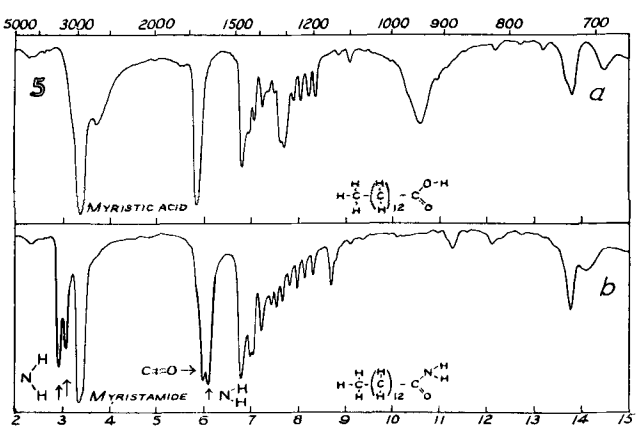

FIG. 8. Infrared absorption spectra of myristic acid and myristamide (25).

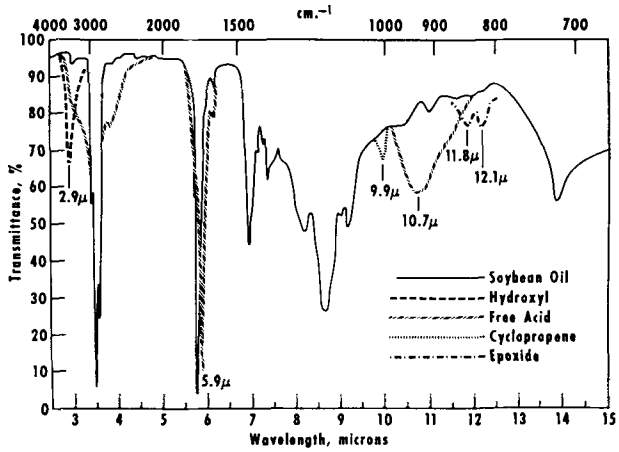

FIG. 9. Characteristic band positions for some chemical structures, superimposed on the spectrum of soybean oil (28).

which has been used both qualitatively and quantitatively for the determination of *cis* double bonds (32). These absorptions are of relatively low intensity, and the 3.3 μm peak is poorly resolved from the saturated C–H stretching bands unless a grating or fluorite prism instrument is used. It seems clear from Fig. 13 that a single *cis* double bond in a fatty acid would be difficult to detect. The characteristic band of the unconjugated *trans* double bond, on the other hand, is strong, sharp and distinct. It is shown in Fig. 14, together with peaks for various conjugated double bond systems (28). Bands of some of the simpler conjugated forms were reported earlier by Jackson et al. (33).

Use With Chromatography

Over the years infrared spectroscopy has been used extensively in conjunction with various forms of chromatography—gas-liquid, liquid-solid and thin-layer. Either a mixture that is too complex for direct infrared analysis can be separated into simpler fractions chromatographically, or unknown fractions obtained by chromatography can be identified by infrared. In gas chromatography it is usually a matter of identification, since quantitation has already been done. Except in liquid column chromatography, micro techniques are required. Methods are available for collecting fractions from both GLC

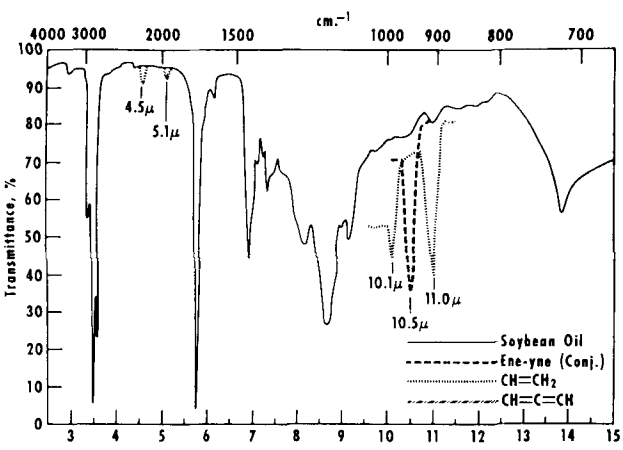

FIG. 10. Characteristic band positions for some unsaturated chemical structures, superimposed on the spectrum of soybean oil (28).

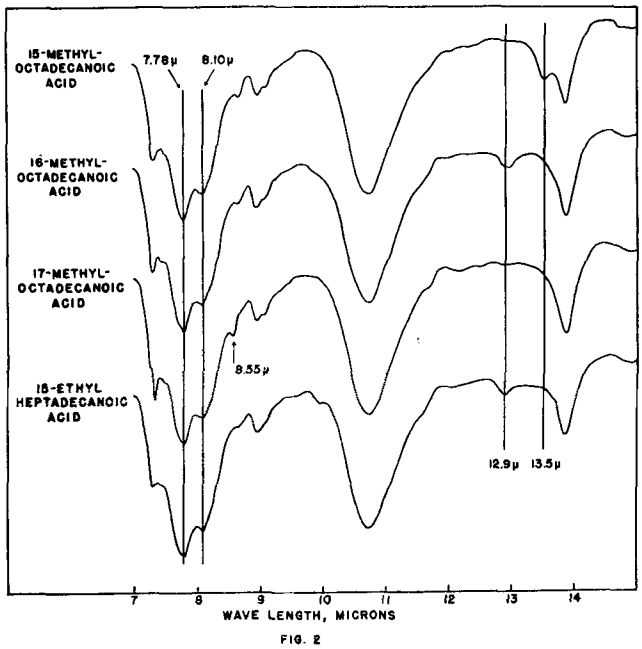

FIG. 11. Terminal ethyl (12.9 μm) and propyl (13.5 μm) group absorptions in long-chain fatty acids (29).

and TLC systems. Identification of fractions has been made spectroscopically by Rouser et al. (34) in thin-layer work with brain and other phospholipids; their work contains a variety of useful phospholipid reference spectra. Morrison et al. (35) used infrared identification of milk phospholipid fractions from both column and thin-layer chromatography. In this connection Nelson (36) has summarized some significant features of phospholipid spectra that are useful for identifying the nitrogenous moiety (Fig. 15).

Quantitative Analysis

Quantitative analysis is usually based on intensity measurement of a relatively strong band. If a pure substance is being measured, the strongest band gives the greatest sensitivity. If the compound being measured is in a mixture, the selected absorption band should be as free as possible from overlapping absorptions of other components. If all of the compounds in a mixture are being determined, each should have a relatively strong band at some position where the other components do not absorb very much. With these guidelines, it is to be expected that functional group bands, as discussed above, will be preferred for quantitative measurement. Some examples may be cited. Nitriles are determined in long-chain fatty amides using the C–N stretching band at 4.4 μm (37). If the amount of nitrile is small, it is first separated chromatographically on a silica gel column. Azelaaldehydic derivatives are measured by means of the aldehydic C–H absorption at 3.7 μm (38). The cyclopropene band at 9.9 μm serves to measure fatty acids containing this group (39). In the near infrared, bands at 1.46 and 2.07 μm were shown to be proportional to hydroperoxide content of autoxidized methyl oleate, with the implication

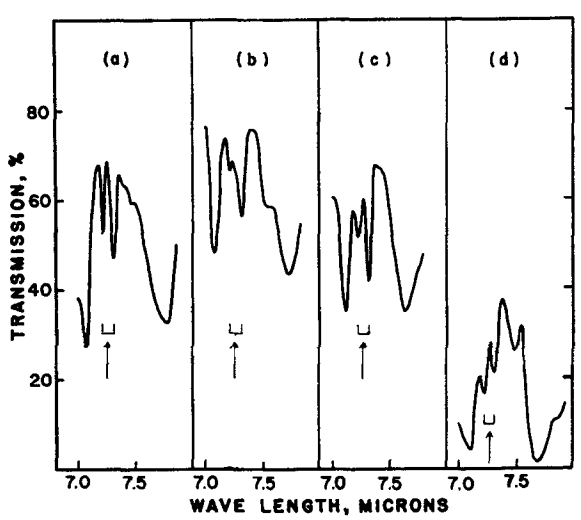

FIG. 12. Splitting of the methyl group absorption band in the infrared spectrum of fatty acids containing two methyl groups on the same carbon atom. (a) 17-methyloctadecanoic acid; (b) 2,2-dimethyloctadecanoic acid; (c) 3,3-dimethyloctadecanoic acid; (d) isovaleric acid (29).

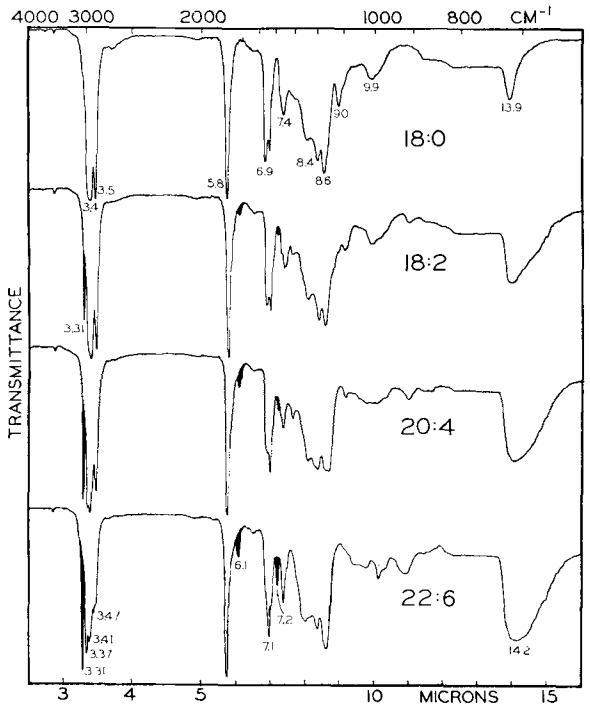

FIG. 13. Infrared absorption spectra of methyl esters containing 0, 2, 4 and 6 unconjugated *cis* double bonds. Double bond absorptions are indicated at 3.31, 6.1, 7.2 and 14.2 μm (7).

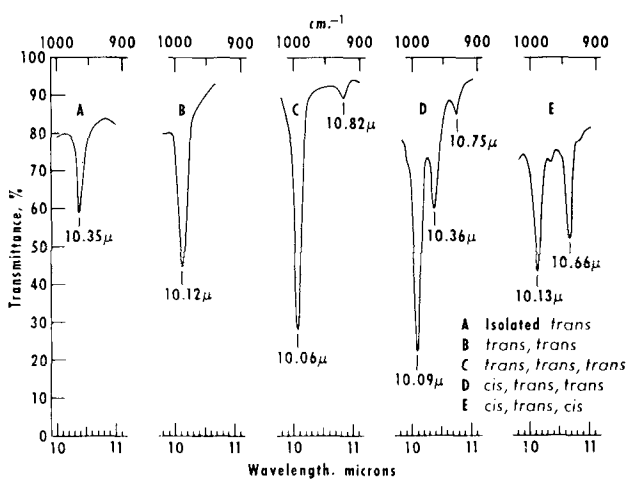

FIG. 14. Absorption bands of unconjugated *trans* double bonds and various conjugated double bond structures (28).

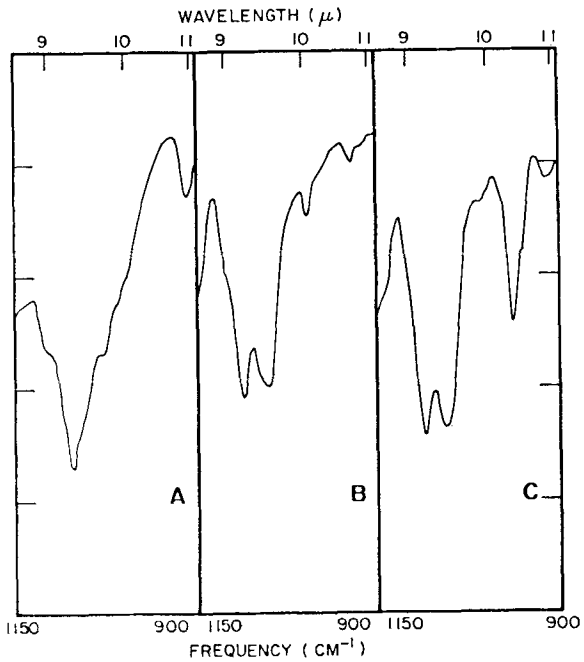

FIG. 15. Absorption patterns related to nitrogenous moieties of some phospholipids. A, phosphatidyl ethanolamine; B, N, N-dimethyl phosphatidyl ethanolamine; C, phosphatidyl choline (36).

that these bands can be used for quantitative measurement in fats and oils (40).

Cis and Trans Double Bonds

One of the best known infrared quantitative methods in the chemistry of fats and oils is the determination of *trans* double bonds. A great number of publications on this method have appeared since the original report by Shreve et al. (18), one of the most recent being by Sreenivasan and Holla (41). This method has survived partly because the separation of *cis* and *trans* isomers by gas chromatography, although possible, is difficult. Since it has been so thoroughly covered in the literature, it might seem that there is little more to be said on the subject. However, in studies of lipids from blood serum and

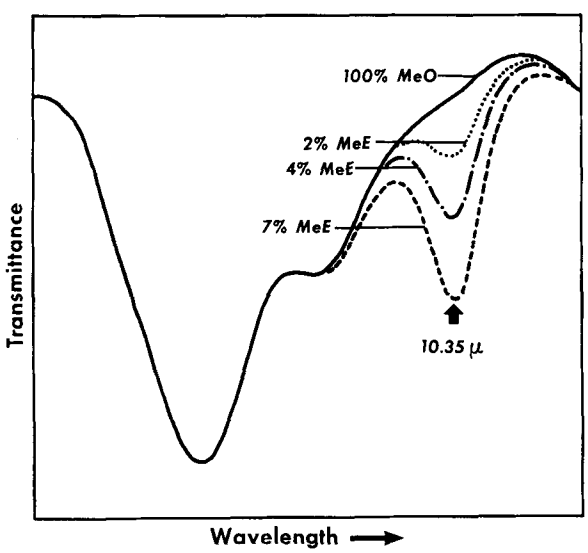

FIG. 16. Infrared spectra (9.5–10.5 μm) of methyl oleate-methyl elaidate mixtures. Carbon disulfide solutions, 15 mg/ml, 3.5 mm microcell (42).

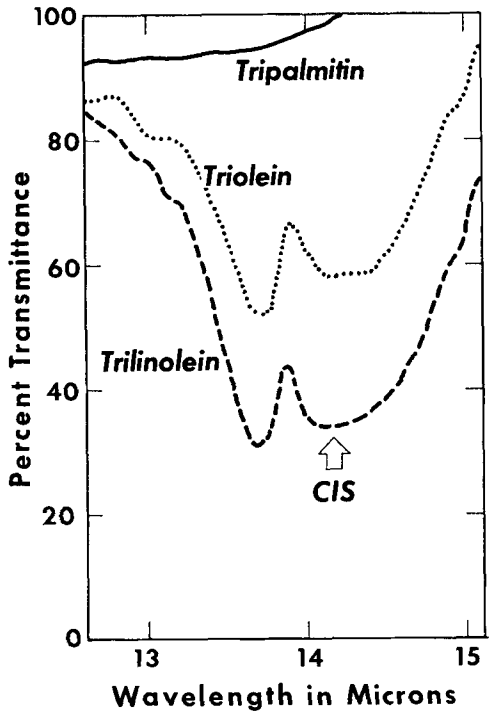

FIG. 17. Differential spectra: calibration standards for measurement of *cis* double bonds. Carbon disulfide solutions, 40 mg/ml, 0.9 mm cells. Tripalmitin solution in reference cell (42).

human depot fat in our laboratory, the procedures may be worthy of comment (42). Perhaps the most interesting feature is that the analysis was done in conjunction with an infrared determination of *cis* double bonds, using the absorption band at 14.2 μm. Serum lipids were converted to methyl esters by methanolysis. The amount of methyl ester per sample was 3 to 5 mg, and it was run at a concentration of 15 mg/ml in CS_2. A microcell was used, with a path length of 3.5 mm, and curves were recorded from 9.5 to 10.5 μm, and from 13.5 to 14.5 μm on a single-beam spectrophotometer. Calibration curves for the first region are shown in Fig. 16. Fat samples were run directly at 40 mg/ml in CS_2 in a 0.9 mm cell, on a double-beam spectrophotometer, with the same concentration of tripalmitin in the reference cell. Calibration runs for this differential analysis are shown for the 14 μm region in Fig. 17. A curve for human depot fat is given in Fig. 18. Although neither the use of a microcell nor the differential method are extraordinary, the author knows of no reported use of the 14.2 μm band for the quantitative measurement of *cis* double bonds in fatty acids or their derivatives. Other infrared methods for *cis* unsaturation are the near infrared method of Holman et al. (32), and the use of the 3.3 μm stretching band as suggested by Sinclair et al. (21). The latter was proposed at a time when the high resolution required was not generally available; otherwise it might have been more widely adopted.

Lipid Classes

Quantitative measurements have been made on serum lipid fractions separated by silicic acid chromatography (43), the components being cholesteryl esters, cholesterol, triglycerides, unesterified fatty acids and phospholipids. Schwartz et al. (44) analyzed tissue lipids in a similar way, using the pressed-pellet sampling technique. A method for the

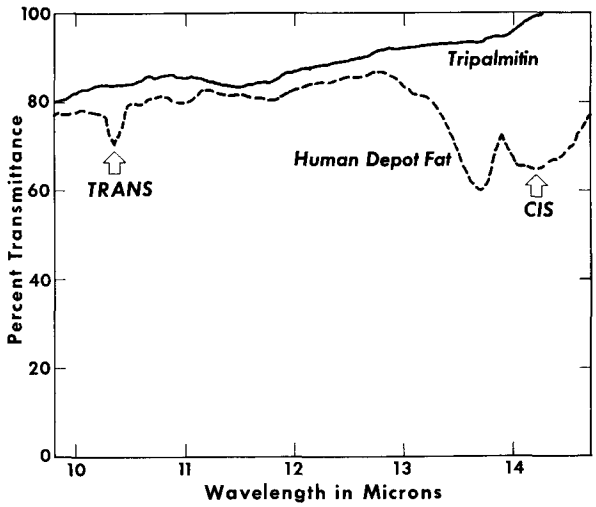

FIG. 18. Differential spectra: fat solutions in carbon disulfide, 40 mg/ml, 0.9 mm cells. Upper: tripalmitin vs. tripalmitin. Lower: deposited fat vs. tripalmitin (42).

determination of serum triglycerides was based on infrared analysis of the appropriate fraction separated by thin-layer chromatography (45).

In our special concern for the analysis of serum lipids, we have developed a two-component infrared method for the simultaneous determination of triglycerides and cholesteryl esters (46). This is carried out on a serum lipid extract from which phospholipids have been removed by adsorption. Under normal circumstances the unesterified fatty acids present in serum do not interfere; and the phospholipid-free mixture has two major components absorbing in the ester carbonyl region. These are the triglycerides and cholesteryl esters, whose absorption bands are shown in Fig. 19. If absorbances of the mixture are measured at the two component peak positions, it is possible to calculate concentrations of the components by the two linear equations:

$$(1a)\ C_1 = K_1 A\nu_1 + K_2 A\nu_2$$
$$(1b)\ C_2 = K_3 A\nu_1 + K_4 A\nu_2$$

in which C_1 and C_2 are the concentrations, $A\nu_1$ and $A\nu_2$ are the absorbances, and the K's are constants calculated by matrix inversion from calibration data for the pure reference compounds. This method has been quite satisfactory, but it does require fairly high resolution (a grating is more than adequate) and good wavelength reproducibility. Measurements have been made at manual instrument settings rather than from a recorded curve.

An instrument has been developed to carry out the triglyceride-cholesteryl ester analysis automatically (47). It consists of a spectrophotometer, computing circuitry, a printing digital voltmeter, and a programming unit. The spectrophotometer is a small nonrecording grating instrument, modified from manual to motor-actuated frequency setting. Once a sample is placed in the instrument, an automatic sequence of measurements is begun. According to the programmed sequence, an absorbance is first measured at ν_1, multiplied by K_1, and the product is stored in a capacitor. The frequency setting is then changed to ν_2, the absorbance there is measured and multiplied by K_2, and the digital voltmeter reads the difference, $K_1 A\nu_1 - K_2 A\nu_2$. (K_2 in eq. 1a is negative.) The result is displayed on the digital voltmeter and also printed out on paper tape as C_1. In a

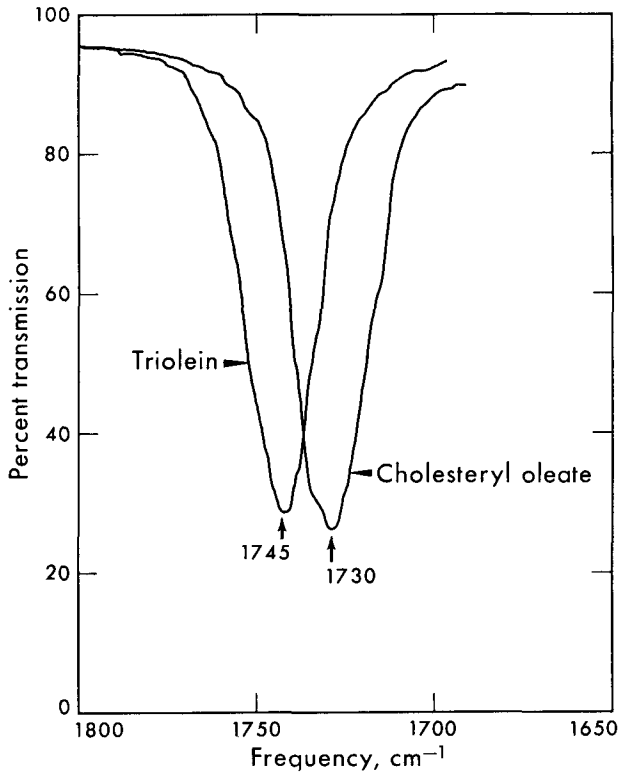

FIG. 19. Ester carbonyl absorption bands of triglycerides and cholesteryl esters at approximately equivalent fatty acid concentrations. Frequency scale expanded to five times normal (46).

similar way C_2 is obtained, starting with the absorbance measurement at ν_2. This time it is multiplied by K_4 and stored. The frequency setting is changed back to ν_1, the absorbance remeasured and multiplied by K_3, and the difference $K_4 A\nu_2 - K_3 A\nu_1$ is printed out at C_2 (K_3 in eq. 1b is also negative). This cycle is completed in about 1 min. Results obtained with the prototype instrument were adequate, but calibration was difficult to maintain because of mechanical instability of the grating mount. With a better monochromator this type of apparatus could be very useful; presumably it could be adapted for other two-component analyses, e.g., triglyceride-fatty acid, lecithin-sphingomyelin, etc.

Computer Applications

It has been indicated that the use of computers in conjunction with infrared spectroscopy is one of the most significant current trends. This has been greatly facilitated by the availability of digital data recorders, by means of which a spectral curve can be recorded on punched cards, punched paper tape or magnetic tape as a sequence of transmittance values. Data are thus provided in a suitably coded form that can be handled directly by the computer. Several uses of the computer in connection with infrared spectra are indicated, including background and frequency correction, peak location, determination of integrated band intensities, and resolution of overlapping bands (48). Applications such as these comprise a separate topic in this symposium, and will not be discussed in detail here. However, in our laboratory we are investigating some specific lipid analyses using some of these techniques, and it is pertinent to describe briefly the nature of these efforts.

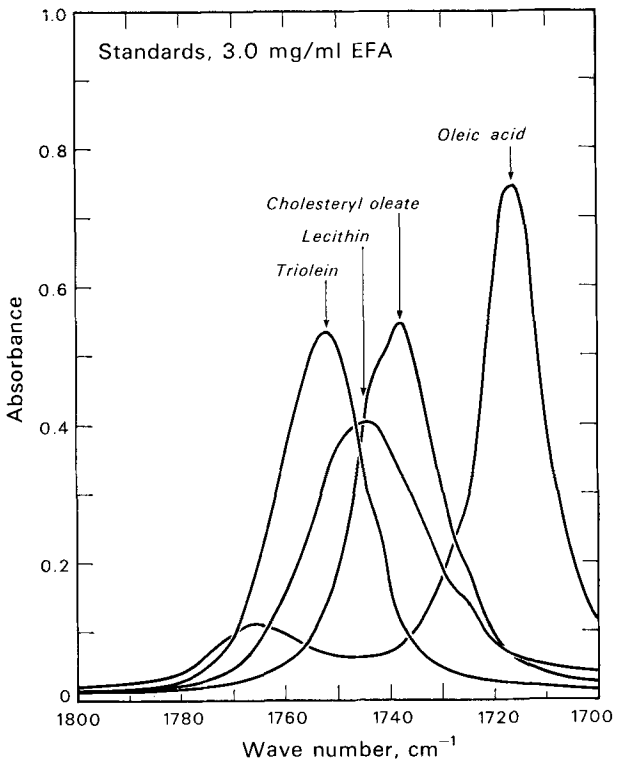

FIG. 20. Carbonyl absorption bands of representative serum lipids. Concentrations in each case adjusted to a fatty acid content of 3.0 mg/ml (CCl_4 solution). Absorbing path length is 1.0 mm (49).

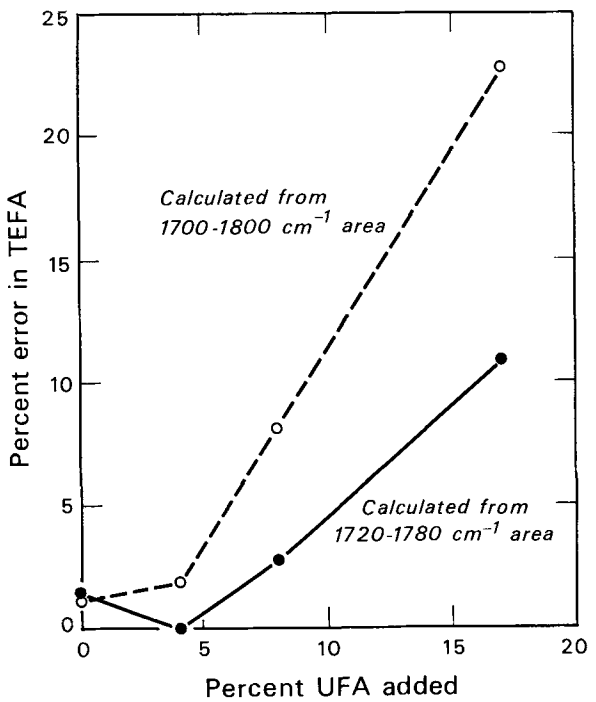

FIG. 21. Relative error in total esterified fatty acid determination as a function of unesterified fatty acid content. – – – –, calculated from 1690–1790 cm^{-1} area. ————, calculated from 1710–1770 cm^{-1} area. Frequencies corrected from those shown in the figure (49).

Integrated Band Intensities

Our initial use of the computer has been to investigate some integrated band intensities and to assess their potential usefulness in the analysis of serum lipids (49). Spectral measurements were made on a Perkin-Elmer Model 421 grating spectrophotometer and recorded on punched paper tape. A particular determination that has been considered is that of total esterified fatty acids (TEFA) based on the ester carbonyl absorption band. As it is usually seen in the serum lipid spectrum, this absorption appears as a single band, but it is actually a composite of overlapping bands of the three main ester types that occur in serum. These bands are shown individually (and on an expanded scale) in Fig. 20, together with the unesterified carboxyl carbonyl band of oleic acid. Although the curve for unesterified fatty acid is shown for a concentration equivalent to that of the esters, it is normally present in serum at a level below 10% of the total fatty acids. It may be noted that for the three ester types there is a frequency at approximately the lecithin maximum where the absorptivities are all very nearly the same. This observation has been used in an earlier method (50) as a means of determining the total esterified fatty acids in serum lipid extracts. However the measurement at a single frequency is highly dependent on the mechanical reproducibility of the instrument, and it is subject to increasing error as the composition of the mixture is weighted toward one extreme or the other. On the assumption that integrated intensities are less subject to these errors, we have determined the integrated carbonyl band areas for various lipids, including those representative of the classes present in serum. The calculation of area is based on absorbances recorded at each 0.5 cm^{-1} interval from 1690 cm^{-1} to 1790 cm^{-1} and multiplying the sum by 0.5, i.e.:

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

Values obtained in this way are empirical and do not represent true integrated intensities. The resulting data for lipids are summarized in Table I. The constancy of B/C for each ester type is an indication of linearity with concentration; and the constancy of B/C_{FA} (last column, Table I) over all four ester classes shows that the band area per ester group is the same, regardless of ester type. Limited data on the analysis of mixtures indicates that in the absence of free fatty acids the determination of total esterified fatty acids from band areas is accurate to about ±2%.

In serum lipids the presence of a small amount of unesterified fatty acids must be taken into account. The fatty acid absorption is a little stronger than the ester band, as is apparent from Fig. 20 and the data in Table I. Its peak position is sufficiently well separated from those of the esters so that it can be resolved from them, but in terms of area there is considerable overlap. An obvious partial remedy is to choose an integration interval that excludes as

TABLE I

Carbonyl Band Areas for Representative Lipids (1690–1790 cm^{-1})

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

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

TABLE II
Absorptivities of Various Lipids in the CH_2 Bending Band

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

[a] α = Absorbance per mg/ml.
[b] β = Band area per mg/ml.

much as possible of the unesterified fatty acid band while still maintaining constancy of the ester band for the various ester types. As a test of this, some prepared mixtures of triolein and cholesteryl oleate containing various added amounts of oleic acid were analyzed, and the results calculated separately for two different integration intervals: 1690–1790 cm^{-1} and 1710–1770 cm^{-1}. For both cases the error in TEFA is plotted as a function of per cent added fatty acid; these graphs are shown in Fig. 21. By using the narrower interval the error is kept below about 5% as long as the unesterified fatty acid content does not exceed about 10%. It seems possible that two-component analyses of esters and free fatty acids might be developed, using areas of appropriately selected sub-intervals.

Another lipid determination in which we have previously used peak heights is that of total lipids in serum, based on the CH_2 bending absorption at about 1460 cm^{-1}. Using a weighted average of the absorptivities at this frequency for the individual lipid constituents, this analysis for total mixed lipids has given results in agreement with gravimetric determinations to about ±3%. It is also somewhat dependent on constancy of sample composition, however, and wide variations in the degree of unsaturation can cause larger errors. Therefore the use of band areas has been investigated as an alternate approach. The CH_2 bending bands of the principal serum lipids

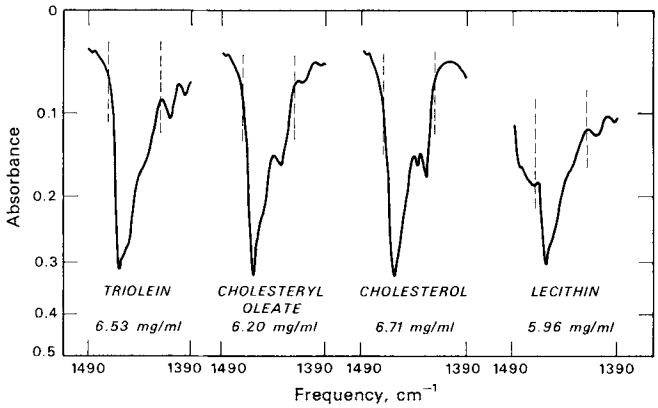

FIG. 22. CH_2 bending bands of representative serum lipids. Roughly comparable concentrations in CCl_4. 1.0 mm cell.

are shown in Fig. 22 at roughly comparable concentrations, over the frequency interval 1390–1490 cm^{-1}. Peak absorptivities (α) as well as integrated absorptivities (β) are given in Table II. The effect of unsaturation is illustrated by the inclusion of data for different triglycerides and different cholesteryl esters. Values of β for two different frequency intervals show that constancy over the whole set of lipids can be improved by a trial and error procedure of finding the best interval. Using the area between 1420 cm^{-1} and 1470 cm^{-1}, calculated total serum lipid values again agreed with gravimetric results to about ±3%. This is comparable in accuracy to the peak absorbance method, but the calibration data indicate that the area method is less susceptible to fluctuations in degree of unsaturation.

Least-Squares Curve Fitting

Another procedure which requires the arithmetical capacity of the computer is that of matching the absorption curve of a mixture to a sum of the contributions of its components. This method was proposed in 1957 by Rogoff (51), who described specialized equipment for this kind of infrared spectral analysis. In current practice the use of digital recorders and general-purpose computers makes this approach feasible. Essentially the computer is asked

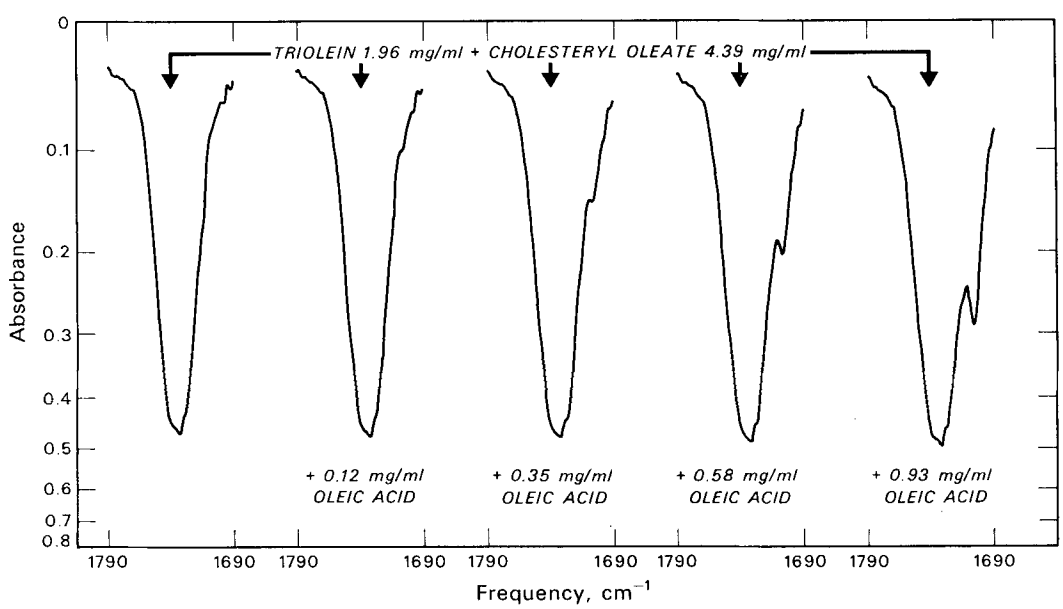

FIG. 23. Carbonyl absorption of a mixture of triolein and cholesteryl oleate containing various amounts of oleic acid. CCl_4 solution, 1.0 mm cell.

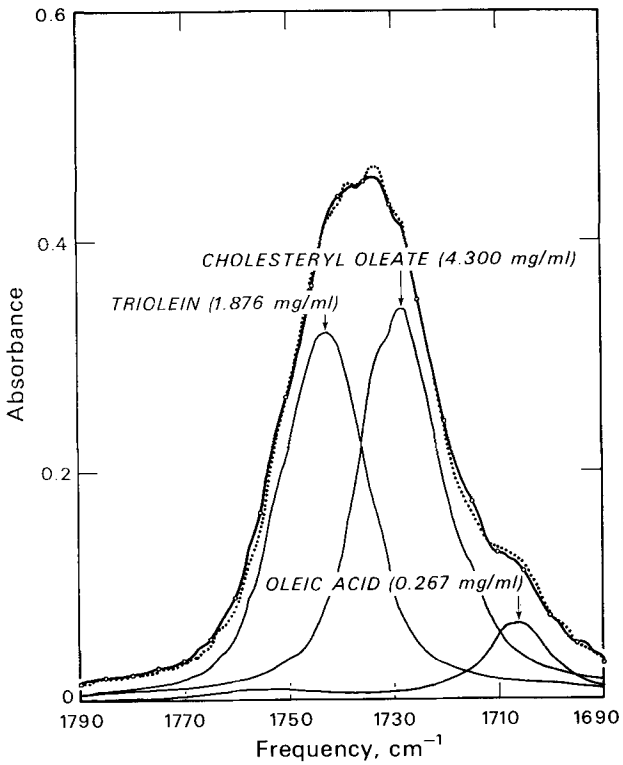

FIG. 24. Dotted curve is a computer plot of Curve 3, Fig. 23. Approximately superimposed solid curve is the summation of individual lower curves, which have been calculated by a least squares method.

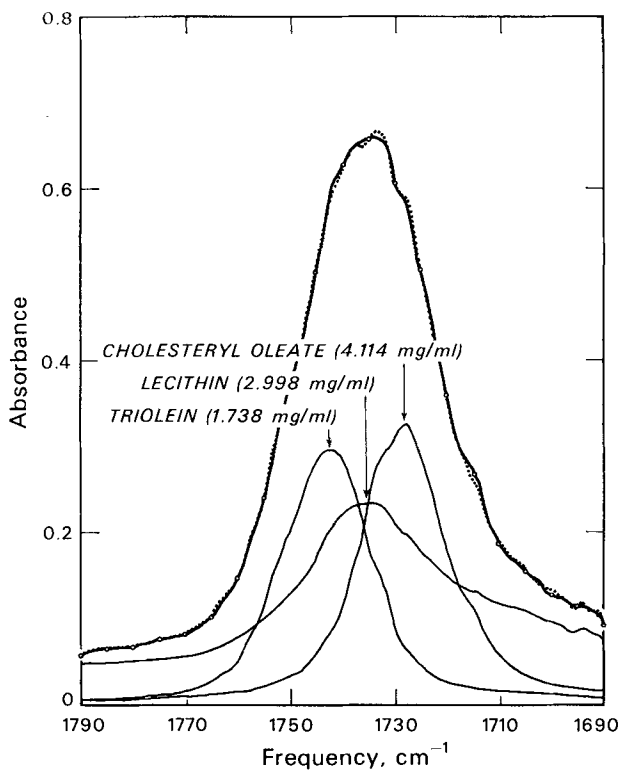

FIG. 25. Three component mixture: computer plot of recorded curve (dotted) compared with summation of calculated component curves (indicated).

to find the concentration of each component for the condition that at every point on the observed curve, the absorbance is equal to the sum of absorbances of the individual components. The actual criterion, of course, is a least-squared-error fitting of the summed curve to the observed curve.

Our beginning attempts to use this method have also been directed to the problem of analyzing mixed lipids from blood serum. Specifically we have used the carbonyl absorptions and have sought in some instances to account for unesterified fatty acid as a component of the mixture. Preliminary results are promising in mixtures from which lecithin is excluded. Figure 23 shows spectrophotometer curves for a prepared mixture of triolein and cholesteryl oleate to which increasing amounts of oleic acid have been added. Under these conditions of resolution the carboxyl peak at about 1710 cm^{-1} becomes discernible when the free fatty acid content reaches about 10% of the total esterified fatty acid content. Analysis of the third curve in Fig. 23 by the computer gave the results shown in Fig. 24. Here are given the curves for individual components at their calculated concentrations, the sum of these individual curves, and the recorded curve of the mixture. The summation curve and the recorded curve are closely superimposed. Analytical results for all five mixtures are given in Table III. Although there are some small systematic errors that have not been accounted for, the triolein and cholesteryl ester values remain reasonably constant; it appears that with suitable adjustments a satisfactory determination of free fatty acids can be developed. One of the problems is that the fatty acids occur in relatively small proportions and the measured absorbances in the vicinity of their peak are correspondingly small.

We have attempted to include phospholipids in a three-component analysis with triglycerides and cholesteryl esters; the preliminary results are encouraging. Figure 25 shows the component curves, their resultant and the recorded curve of the mixture. The degree of correspondence of the latter two curves is very good, but the calculated concentrations differ from known concentrations by 3%, 7% and 12%. It has not been determined whether these errors are due to poor calibration or to the compounding of measurement errors, or both. It was observed that the initial results were even more erratic, but that they were improved when the lecithin curve was recalibrated using the sample from which the mixture was made. The next step will be to analyze the total mixture containing the three types of esterified fatty acids plus unesterified fatty acids. What accuracy will be attainable remains to be established, but this method should prove useful in analyzing mixed lipids.

TABLE III

Analysis of Lipid Mixtures by Least-Squares Curve Fitting (conc. in mg/ml)

Sample	Prepared Composition TO	CO	OA	Composition by Analysis TO	CO	OA
1	1.96	4.39	0.00	1.75	4.44	0.006
2	1.96	4.39	0.12	1.87	4.28	0.07
3	1.96	4.39	0.35	1.88	4.30	0.27
4	1.96	4.39	0.58	1.87	4.35	0.48
5	1.96	4.39	0.93	1.87	4.36	0.83

TO = Triolein
CO = Cholesteryl Oleate
OA = Oleic Acid

ACKNOWLEDGMENTS

L. C. Jensen programmed the computer calculations. This work was carried out under the auspices of the U.S. Atomic Energy Commission.

REFERENCES

1. Thompson, H. W., J. Chem. Soc. 328, (1948).
2. Baker, A. J., T. Cairns, G. Eglinton and F. J. Preston, "More

Spectroscopic Problems in Organic Chemistry," Heyden and Son, Ltd., London, 1967.
3. Paschke, R. F., L. E. Peterson and D. H. Wheeler, JAOCS *41*, 723 (1964).
4. Sasaki, S., H. Abe and T. Kato, Chem. Eng. News *46*, 44 (1968).
5. Gebbie, H. A., in "Advances in Quantum Electronics," Columbia University Press, New York, 1961, pp. 155–163.
6. Coleman, I., and M. J. D. Low, Spectrochim. Acta *22*, 1293 (1966).
7. Holman, R. T., in "Progress in the Chemistry of Fats and Other Lipids," Vol. 9, Pergamon Press Ltd., London, 1966, pp. 71–75.
8. Herr, K. C., and G. C. Pimentel, Appl. Optics *4*, 25 (1965).
9. Anderson, D. H., and O. E. Miller, J. Opt. Soc. Am. *43*, 777 (1953).
10. Cole, A. R. H., and R. N. Jones, J. Opt. Soc. Am. *42*, 348 (1952).
11. Schiedt, U., and H. Reinwein, Z. Naturforsch. *7b*, 270 (1952).
12. Hatch, F. T., N. K. Freeman L. C. Jensen, G. R. Stevens and F. T. Lindgren, Lipids *2*, 183 (1967).
13. Ambrose, E. J., and W. E. Hanby, Nature *163*, 483 (1949).
14. Susi, H., and A. S. Jahn, JAOCS *43*, 479 (1966).
15. Chapman, D., "The Structure of Lipids by Spectroscopic and X-Ray Techniques," John Wiley and Sons, New York, 1965.
16. Chapman, D., JAOCS *42*, 353 (1965).
17. Shreve, O. D., M. R. Heether, H. B. Knight and D. Swern, Anal. Chem. *22*, 1498 (1950).
18. Shreve, O. D., M. R. Heether, H. B. Knight and D. Swern, Anal. Chem. *22*, 1261 (1950).
19. O'Connor, R. T., E. T. Field and W. S. Singleton, JAOCS *28*, 154 (1951).
20. Sinclair, R. G., A. F. McKay and R. N. Jones, J. Am. Chem. Soc., *74*, 2570 (1952).
21. Sinclair, R. G., A. F. McKay, G. S. Myers and R. N. Jones, J. Am. Chem. Soc. *74*, 2578 (1952)
22. Jones, R. N., A. F. McKay and R. G. Sinclair, J. Am. Chem. Soc. *74*, 2575 (1952).
23. Meiklejohn, R. A., R. J. Meyer, S. M. Aronovic, H. A. Schuette and V. W. Meloch, Anal. Chem. *29*, 329 (1957).
24. Kirby, E. M., M. J. Evans-Vader and M. H. Brown, JAOCS *42*, 437 (1965).
25. Scott, R. B., and J. M. Vandenbelt, "The Beckman Bulletin, No. 14," Beckman Instruments, Inc., Fullerton, California 1954.
26. Colthup, N. B., J. Opt. Soc. Am. *40*, 397 (1950).
27. O'Connor, R. T., JAOCS *33*, 1 (1956).
28. Wolff, I. A., and T. K. Miwa, JAOCS *42*, 208 (1965).
29. Freeman, N. K., J. Am. Chem. Soc. *74*, 2523 (1952).
30. Guertin, D. L., S. E. Wiberley, W. H. Bauer and J. Goldenson, Anal. Chem. *28*, 1194 (1956).
31. Keeney, P. G., JAOCS *39*, 304 (1962).
32. Holman, R. T., S. Ener and P. R. Edmondson, Arch. Biochem. Biophys. *80*, 72 (1959).
33. Jackson, J. E., R. F. Paschke, W. Tolberg, H. M. Boyd and D. H. Wheeler, JAOCS *29*, 229 (1952).
34. Rouser, G., G. Kritchevsky, D. Heller and E. Lieber, JAOCS *40*, 425 (1963).
35. Morrison, W. R., E. L. Jack and L. M. Smith, JAOCS *42*, 1142 (1965).
36. Nelson, G. J., Lipids *3*, 104 (1968).
37. Buswell, K. M., and W. E. Link, JAOCS *41*, 717 (1964).
38. Anders, D. E., and E. H. Pryde, JAOCS *43*, 305 (1966).
39. Bailey, A. V., G. J. Boudreaux and E. L. Skau, JAOCS *42*, 637 (1965).
40. Holman, R. T., C. Nickell, O. S. Privett and P. K. Edmondson, JAOCS *35*, 422 (1958).
41. Sreenivasan, B., and K. S. Holla, JAOCS *44*, 313 (1967).
42. Freeman, N. K., "Semiannual Report," Donner Laboratory, Lawrence Radiation Laboratory, UCRL-9897, 1961, p. 96.
43. Freeman, N. K., F. T. Lindgren, Y. C. Ng and A. V. Nichols, J. Biol. Chem. *227*, 449 (1957).
44. Schwartz, H. P., L. Dreisbach, R. Childs and S. V. Mastrangelo, Ann. N. Y. Acad. Sci. *69*, 116 (1957).
45. Krell, K., and S. A. Hashim, J. Lipid Res. *4*, 407 (1963).
46. Freeman, N. K., J. Lipid Res. *5*, 236 (1964).
47. Freeman, N. K., E. Lampo and A. A. Windsor, JAOCS *44*, 1 (1967).
48. Eddy, C. R., and J. S. Showell, JAOCS *43*, 643 (1966).
49. Freeman, N. K., "Semiannual Report," Donner Laboratory, Lawrence Radiation Laboratory, in press.
50. Freeman, N. K., Ann. N. Y. Acad. Sci., *69*, 131 (1957).
51. Rogoff, M., Ann. N. Y. Acad. Sci., *69*, 27 (1957).