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PART I

The Chemical and Biological Assay of Essential Fatty Acids[1]

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Abstract

After defining and developing the background of essential fatty acids, the various chemical, biological and physical methods which have been used for their assay will be reviewed. Specific recommendations will be made regarding techniques in each method category for natural and processed fats. Special attention will be given to the distinction between the essential fatty acids and the other polyunsaturated acids.

Introduction

In 1929 Burr and Burr presented evidence of the essential nature of linoleic acid for the growth of rats (5). The nature of the fatty acids required for the proper growth and health of experimental animals and man was uncertain. Without experimental evidence it was assumed that linoleic and linolenic acids were both effective. As data accumulated, a minor role was assigned to linolenic acid. It was finally resolved that the nutritive values of the latter acid, if it had special nutritive properties at all, were of a different nature from those of linoleic acid.

Studies with rats, growing chicks, and hens have demonstrated various inter-relationships, especially that of linoleic to arachidonic acids. The mechanisms of these conversions were finally elucidated by Mead and co-workers (36), who demonstrated that linoleic acid is dehydrogenated, acetate added, and the chain again dehydrogenated in the formation of arachidonic acid.

Recently a special role for the polyunsaturated fatty acids has been demonstrated in maintaining normal blood cholesterol values and in preventing the deposition of excess cholesterol in the intima of arteries, especially the coronary artery. It has become quite clear, however, that the mechanisms for the lowering of serum cholesterol, or of the control of cholesterol synthesis by the polyunsaturated fatty acids are not the same as the mechanism whereby linoleic and arachidonic acids assist the growth and maintenance of healthy tissues. Fish oil fatty acids, inactive in preventing classical essential fatty acid deficiency symptoms, are quite active in maintaining normal blood cholesterol levels.

These considerations indicate a need for definition of the term, "essential fatty acids." Although sometimes used synonymously with the term "polyunsaturated fatty acids," such usage is both inaccurate and confusing. The term "essential fatty acids" should be reserved for those acids required in the maintenance of normal growth, reproduction, and skin permeability. These properties are characteristic of the acids in the "linoleic acid series" (linoleic, γ linolenic, and arachidonic), which have a common terminal structure, $CH_3(CH_2)_4\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-CH_2-\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-$ [i.e., double bonds in the 6,7 and 9,10 positions counting from the terminal end (65)]. An additional requirement for biological activity is that both of these double bonds must be in *cis* configuration. The mechanism of action of the linoleic acid series appears to resemble that of vitamins and, indeed, these have often been referred to as vitamin F (8). Although the functional mechanisms of these acids are not known, they might possibly play some role in enzyme systems.

The function of fatty acids of the linolenic acid series, $CH_3CH_2\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-CH_2\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-CH_2\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-$, on the other hand, may be structural rather than metabolic cofactors. Thus, the anti-atherosclerotic activity of polyunsaturated fatty acids in the prevention of high cholesterol accumulation or synthesis, has been ascribed to their influence on permeability of cell walls and transport of cholesterol through the liver into the intestine.

Whether or not the polyunsaturated fatty acids are catabolized differently than the saturated fatty acids

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is not known. Probably unsaturated acids are preferentially stored and metabolized, depending upon their particular structure, and when catabolized, follow pathways differing from those taken by saturated fatty acids. Certainly they play different roles in the synthesis of triglycerides, phospholipids, and cholesteryl esters.

As the complexity of the metabolic and nutritional patterns of the polyunsaturated fatty acids has been realized, the need for analytical techniques for their determination has increased.

Fortunately, progress in column, paper, thin-layer, and gas chromatography of fatty acids, has also been developing rapidly. Other instrumental techniques, such as ultraviolet and infrared spectrophotometry and nuclear magnetic resonance, have found use in this field.

An enzymatic technique has been developed (32), and methods improved for the oxidation of polyunsaturated fatty acids (6) which enable one to more accurately determine the position of double bonds in the chain.

While it is true that significant advances have been made in the chemical and physical determination of fatty acids, it may occasionally be preferable to use a bio-assay for the determination of essential fatty acids. Bio-assay has the advantage of distinguishing between positional and geometrical isomers, some of which are not physiologically active. Most commonly used physical and chemical procedures will not make this distinction.

Discussion

Biological Assays

Biological assays have shown that the fatty acids of the linoleic acid series are those with the biopotency. A chemical or instrumental determination of the fatty acids of this series is relatively simple when dealing with vegetable oils of limited fatty acid composition. It becomes more difficult when dealing with animal fats containing complex mixtures of unknown composition, or with hydrogenated fats containing biologically inert geometrical and positional isomers.

Although with a combination of infrared spectrophotometry, ozonolysis and gas chromatography one may be able to determine the level of true essential fatty acids in such fats, the method of choice is certainly the bio-assay. The restricted water intake method of Thomasson (65, 66) is probably the shortest and most accurate of the biological assay techniques. There are indications that assays permitting unrestricted water intake (11) may be misleading in that normal growth may be obtained, even though skin symptoms are quite abnormal.

Water Balance Method. The bio-assay of Thomasson (65, 66) is based on the fact that deficient animals lose more water through their skins than healthy controls, and therefore have a higher requirement for it. At a water intake of 14 ml per day the maximum difference in weight gain at 9 weeks between control and deficient animals is the criterion for the level of essential fatty acids.

Male rats, 21 days old, which had received the Sherman diet during their lactation period, are placed on the essential fatty acid deficient ration. The fat soluble vitamins are administered in the form of a prophylactic dose, at the beginning of the experiment and subsequently every 4 weeks.

Water is supplied *ad libitum* for the next 14 days. On the 15th day the animals are weighed and placed in separate cages. The drinking water is limited to 14 ml per animal per day for a period of 3 weeks. The water may be rationed by hand or by various automatic devices (65).

This first 5 weeks constitutes the depletion period. This is followed by a 4 week test period. At the beginning of this period the animals are weighed and equally distributed according to their body weights into the various groups. The animals receive 0.2 ml of the test or standard oil per day for 5 days each week. Twelve animals are used for each level of each standard or unknown oil.

Safflower or similar oils are used as a standard rather than the pure acid. The activity of an oil equivalent to 10 mg of linoleic acid is taken as one biological unit. Since it has been demonstrated that there is a linear correlation between the growth response and the logarithm of the dose administered, it is only necessary to use two levels of the standard and the unknown oils. The standard levels are the amounts of oil equivalent to 5 and 25 mg of linoleic acid respectively made up to 0.2 ml with completely hydrogenated coconut fat. The unknown oil is also fed at two levels, the larger being five times that of the smaller. The amount of unknown oil required for an assay is equivalent to about 1.5 g of linoleic at the lower level and 7.5–10 g at the higher level.

Symptom Score Method. Holman's symptom score technique (17,19) is designed for relative values only. Individual rats are inspected periodically for symptoms of essential fatty acid deficiency: scaliness of feet, scaliness of tail, and roughness of haircoat.

Insect Assay. The insect assay of Vanderzant, Kerur, and Reiser (68) though not completely developed, requires very small amounts of material and considerably less time than the rat methods. Moths reared through the larval stage on diets devoid of essential fatty acids cannot emerge from the pupal cases. The percentage of moths which do emerge appears to be proportional to the level of essential fatty acids.

Chemical Procedures

Polybromide Numbers. Because the recently developed spectrophotometric and chromatographic methods are far more accurate and simple, the polybromide number procedure is no longer of much value. The method depends upon the relative insolubilities (1,58,4) of the di-, tetra-, hexa-, and octabromides in various organic solvents. Since little more than half the theoretical yields are obtained from natural fats, empirical conditions have to be established.

Isotope Dilution Combined with Tetrabromides. van Beers, Sparreboom and Keppler (67) devised a method for the determination of *cis, cis* linoleic acid in the presence of its geometrical isomers. This method is a combination of isotope dilution and tetrabromide number techniques.

Thiocyanogen Number. Kaufmann (24,25) is responsible for the observation that the thiocyanogen reagent, which was initially shown to react with unsaturated hydrocarbons, also reacts with unsaturated fatty acids. It was reported that it reacts with the one double bond of oleic acid, with one of the two of linoleic acid, and with two of the three of linolenic acid. Although later work demonstrated that these ratios are not strictly true, the method was rapidly standardized when combined with iodine value (I.V.) for the empirical assay of mixtures of unsaturated fatty acids (42). This method is still of value in the commerce of fats and oils, and in following the course

of hydrogenation. However, it is much inferior to the spectrophotometric and gas chromatographic techniques. Little is known about the role played by positional and geometrical isomers so that the procedure is of no value in the determination of essential fatty acids in the presence of other isomers.

Enzymatic Method

An enzymatic method has been devised by MacGee (32) for the determination of the total *cis*-methylene interrupted polyunsaturated fatty acids. This method depends on the formation of conjugated diene hydroperoxide by the enzyme lipoxidase in atmospheric oxygen. It does not distinguish between fatty acids of the linoleic or linolenic acid series, nor between fatty acids varying in chain length.

Physical Methods

Ultraviolet Spectrophotometry. In 1937 Moore (38) observed that methylene interrupted double bonds in polyunsaturated fatty acids become partially conjugated upon prolonged saponification. Since conjugated double bonds absorb ultraviolet light at specific wave lengths, whereas methylene interrupted double bonds do not possess this property, empirical conditions were soon found which permitted the assignment of reproducible extinction coefficients to the various isomerized polyunsaturated fatty acids at the wave lengths of maximum absorption. Utilizing the extinction coefficients of individual fatty acids and the iodine numbers of the mixtures, a procedure was developed (37) for measuring polyunsaturated and monounsaturated fatty acids. The saturated acids were determined by difference. In 1944, the method was extended by Beadle and Kraybill (2) to include arachidonic acid.

Since the degree of isomerization varies with a number of factors the method is empirical. The solvent used, the concentration of the alkali, time, temperature, and even the size of the fat or fatty acid sample, must be rigidly standardized. Many different conditions have been recommended by various laboratories. All of these are compromises since no set of conditions have been found which will permit complete isomerization and prevent destruction of some double bond systems (48).

There are also other limitations. The extinction coefficients which are used have been derived from linoleic, linolenic, and arachidonic acids naturally occurring in vegetable fats. In the mixed fats of animal origin, however, there may be also present penta- and hexaenoic acids of different chain lengths. *Trans* isomers are present in such fats as hydrogenated shortenings and the tallows of ruminant animals. Since the extinction coefficients of *trans* acids are not exactly the same as those of *cis*, and those of C_{20} pentaenoic are not the same as those of C_{22} pentaenoic (12), the results of analyses by this method must be interpreted advisedly.

These limitations are especially significant when one is interested in the biologically active essential fatty acids. Since the geometrical and positional isomers are not biologically active, yet are included in the spectral method, the procedure may be quite useless when applied to certain hydrogenated fats in which large amounts of isomers are present. It must be emphasized, therefore, that much judgment must be exercised in the use of the spectral method for the determination of essential fatty acids.

Another possible error in the use of the spectrophotometric procedure is the spurious trienoic and tetraenoic peaks produced by oxidized dienoic and trienoic acids. Although these may not be great, they may give false impressions of the presence of arachidonic and linolenic acids when none are present. Therefore, judgment must be exercised in reporting small amounts of the trienoic and tetraenoic acids in fats where they should not, by other criteria, be expected to be present. In spite of the above uncertainties, with the use of proper judgment the isomerization-spectrophotometric procedures are extremely useful and have done much to advance our knowledge of the chemistry and metabolism of the polyunsaturated acids. Furthermore, they have recently been shown to compare very well with the gas chromatographic procedures (13).

The choice of which spectrophotometric procedure to select depends not only upon the complexity of the unsaturated fatty acid mixture in the sample but on the amount of available sample and the nature of the biological material from which it was obtained. Thus, if one has a large amount of a relatively simple oil in which the only polyunsaturated acid is known to be linoleic, the macro method (43) of the AOCS, Cd 7-58, is the method of choice. For animal fats in which one may wish to determine the unsaturated acids up to and including the hexaenes, the procedure of Herb and Riemenschneider (15) is more satisfactory. For small amounts of isolated animal fats, the micro-modification (14) is the method of choice. One must be aware, however, that although in all probability the great bulk of the dienoic acid in such animal fat is linoleic, and the tetraenoic probably arachidonic, unknown proportions probably are not these. Furthermore, it is still not known to what degree the longer chain, more highly unsaturated, acids belong to the biopotent linoleic acid series. Thus, this method is useless for the assay of essential fatty acids in marine oils.

Functional groups transparent in the near ultraviolet exhibit strong, characteristic absorption bands at shorter wave lengths. In 1945 Rusoff and coworkers (53) examined various fatty acids down to 170 mμ and found useful correlations between their absorption and the number and type of double bonds present. Recent improvements in far ultra-violet spectrophotometers (29) will probably stimulate additional absorption studies in this region.

Near Infrared Spectrophotometry. The application of near infrared spectroscopy may prove to be very useful in parameters of essential fatty acid assays. It may be especially valuable in following the effects of hydrogenation upon the essential fatty acids.

As discussed by Wheeler (69), infrared spectroscopy was first used in oils and fats to determine *trans* double bonds. Holman, however, has demonstrated that isolated *cis* unsaturation also may be detected by a characteristic absorption maximum at 2.15μ (16) and has further developed the technique for direct quantitative assay of *cis* double bond fatty acids (18). This method was expanded by Fenton and Crisler (10).

Unfortunately, the near infrared method measures total *cis* double bonds, and would have to be used in conjunction with other procedures for the assay of linoleic acid or other essential fatty acids.

Nuclear Magnetic Resonance. Nuclear magnetic resonance has found application in the determination of essential fatty acids (62). Although it was found that linolenic and linoleic acids may be assayed in

mixtures, and hydrogenation of the double bond at the 15 position can be followed, the technique has not been studied thoroughly enough to predict its general utility in essential fatty acid assays.

Chromatographic Methods. All of the various chromatographic techniques, adsorption column, reversed-phase column, paper and liquid vapor phase chromatography have been readily adapted to the assay of the longer chain polyunsaturated acids. It should be pointed out however, that the use of chromatography for the quantitative assay of the essential fatty acids in the presence of isomers is still difficult and may require other complementary analytical methods.

Column Chromatography. Adsorption column chromatography has not proven very successful for the quantitative assay of essential fatty acids, and is even limited in its usefulness for the separation of these acids. Riemenschneider and co-workers (52) have reported the successful isolation of methyl linoleate and linolenate from various seed oils with silicic acid, but made no effort to adapt it to a quantitative assay.

Reversed-phase column chromatography has proven much more useful than the more simple adsorption principle but is still not completely successful for the separation of complex mixtures of fatty acids. Unfortunately, the difference in partition coefficient produced by an elongation of the carbon chain by two methylene groups is counterbalanced by the presence of one additional double bond, so that myristic, palmitoleic, linoleic, and arachidonic acids are not well separated, nor are palmitic and oleic. Furthermore, very little work appears to have been done with *cis, trans,* or positional isomers.

The reversed-phase method, however, has been found to be of some value in systems in which preliminary separations into groups are followed by the chromatography of derivatives of the groups. Thus Savary and Desnuelle (54) after separation into groups, oxidized the unsaturated acids of each group to their corresponding polyhydroxy derivatives with dilute alkaline permanganate at low temperature. The di-, tetra-, and hexa-hydroxy derivatives were readily partitioned. Mead (35) and Steinberg and co-workers (59) have hydrogenated (49) inseparable groups (i. e. myristic, palmitoleic, linoleic). The resultant mixture of saturated fatty acids can be readily partitioned by reversed-phase chromatography.

Such techniques must be used advisedly when the quantitative assay of essential fatty acids is sought. The results are quite meaningless where significant quantities of biologically inactive isomers are present.

Paper Chromatography. As a quantitative tool, reversed-phase paper chromatography has advantages over the column. It requires much smaller samples and permits wider adaptation of modifications of both the solid support and the liquid stationary phase. It also is adaptable to many techniques for identification of constituents. It is sparing of time and solvents, of which column chromatography is very demanding. It is also more adaptable to the use of derivatives of the fatty acids and environmental temperature can be better controlled. Finally, it is easier to quantitate by the use of densitometry and other special techniques.

The same limitation that two carbon atoms are equivalent to one double bond applies to paper chromatography as it does to column. This limitation has been partially circumvented in paper chromatography by the use of derivatives. However, this has been only partially successful and, in some cases, as pointed out by Schlenk (55), the formation of isomers during derivative preparations may further complicate the situation.

Schlenk also reported that resolution of saturated and unsaturated acids, and *cis* and *trans* isomers, can be obtained by reducing the temperature, since the saturated and *trans* acids are relatively immobile at 0C or −5C. Kaufmann and Mohr (26) have also utilized the effect of temperature in reducing the migration of saturated acids to develop two dimensional chromatogram at −30C and 120C.

As with column chromatography, the interference due to the balancing effects of chain length and unsaturation has been resolved by paper chromatography of the sample before and after hydrogenation (55).

The use of hydroxy- or bromo-derivatives have been used by a number of investigators for resolving the co-travelers such as palmitic and oleic, or myristic and linoleic. Kaufmann and Nitsch (28) found that stearic, mono-, di-, tri-, and tetrahydroxystearic acids could all be resolved, as could di-, tetra-, and hexabromstearic acids. These authors used cellulose paper impregnated with undecane, with acetic acid-water mixtures as the mobile phase. The acids were visualized by staining with Rhodamine B, the salts with copper ferrocyanide.

Ory, Bickford, and Dieckert (45) have successfully adapted the bromo-derivative technique to silicic acid impregnated glass fiber filter paper.

Inouye and co-workers (20) were able to resolve the problem of super-position by the reversed-phase chromatography of mercuric acetate derivatives of the unsaturated acids, and subsequent development of colored spots by reaction with diphenylcarbazone. They suggested the possibility of quantitative assay of the colored derivative by spectral analysis.

The mercuric acetate, as well as copper and mercury soaps and similar derivatives, were adapted and utilized by Kaufmann and Schnurbusch (27) and by Schmidt (56). Seher (57) also exploited the copper salt technique and Perilä (46) explored the use of silver salts.

Whatever scheme is utilized for the separation of the various fatty acids, it is necessary to locate them on the paper and to identify, and finally to quantitate them.

Densitometric measurements are commonly used. This technique measures both the area and intensity of a spot and, with proper filters, can be used with any color. By the use of the densitometer, one obtains a series of peaks. The area under any particular peak expressed as the percentage of the total area under all peaks, represents the percentage in the sample of this particular substance. If the original chromatogram is difficult to handle, photographs of the spots can be used (45,64).

In the case of silicic acid impregnated glass fiber filter paper, the fractionated lipid may be located by charring with heat after spraying with sulfuric acid (45). The lipid may then be assayed in a specially constructed densitometer (64).

As in the case of column chromatography, there has been very little work done with paper chromatography on the isomers of the unsaturated acids. Schlenk et al. (55) have demonstrated that elaidic and oleic acids do not separate at 31C but may be separated at 0C on paper. As with other assay methods, the determination of the essential fatty acids by paper chromatography, in a simple mixture such as cottonseed oil acids, is quite straight for-

ward, but is uncertain in the presence of significant quantities of isomers.

Thin-layer Chromatography. The technique of coating glass plates with thin-layers of silicic acid and alumina is a fast growing analytical method. Although most of the lipid work has been concerned with the partitioning of essential oils and lipids, some work has been done with fatty acids (33,34,39, 41). The methyl esters of unsaturated and saturated members of homologous and vinylogous series are separated as the mercuric acetate addition compounds and the original ester recovered for further analysis (34,40,47).

Gas-liquid Chromatography. Numerous publications have appeared in which gas-liquid analyses of polyunsaturated acids of natural fats have been reported (3,7,21,22,44,60,61). Farquhar and co-workers (9) have published an excellent description of gas-liquid chromatography application to the analysis of fatty acid mixtures, including menhaden oil.

No one appears to have developed a unified gas-liquid chromatographic system for the determination of the fatty acids in complicated mixtures, such as partially hydrogenated fish oils, containing *cis, trans,* and positional isomers, even though the retention times (and volumes) of some of these have been studied (3,22). Capillary columns have been used for the separation of *cis-trans* isomers (30,31).

If a polar stationary phase is used, the order of elution is that of the chain length, the unsaturated appearing in the order of their degree of unsaturation. Thus, palmitoleic follows palmitic, stearic is followed by oleic, linoleic, and linolenic in that order. These are followed by the 20 carbon series. The 20 carbon saturated acid, arachidic, will coincide with an 18 carbon polyunsaturated acid. The order of the appearance of the constituents is different with the use of non-polar stationary phases, the unsaturated and branched chains moving ahead of the corresponding saturated.

The interpretation of a complex chromatogram becomes more complicated by the fact that on a non-polar stationary phase, the closer to the carboxyl the double bond, the later the compound leaves the column; that conjugated acids have longer retention times (3,22), than methylene interrupted ones; and that *trans* isomers move more slowly than *cis*. However, these differences make the technique useful for the assay of mixtures of the various isomers.

The technique of utilizing both a non-polar and a polar stationary phase for the analysis of mixtures such as one might find in hydrogenated fats is doubtless the best available (9,22,61). Positional and geometrical isomers and branched chain acids were separated and identified on Apiezon L stopcock grease, and the various polyunsaturated acids on polyethylene glycol adipate.

It is possible to isolate fatty esters after gas-liquid chromatography and subject them to various analytical procedures. Hydrogenation to saturation followed by rechromatography may determine the chain length (23). Oxidation and chromatography of the fragments enables one to determine the position of the double bonds (6,23,51). Geometrical isomers can be determined by capillary columns (30,31) or by infrared spectroscopy (10).

In the final analysis, it appears that the bio-assay is certainly best for the determination of total essential fatty acids in complex mixtures, and gas-liquid chromatography or ultraviolet spectral analyses are best for linoleic acid in natural fats except fish oils.

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