Recent Advances in the Determination of the Structure of Fatty Acids via Ozonolysis[1]

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Abstract

Important factors and new techniques in the application of ozonolysis to the localization of double bond positions in unsaturated fatty acids are elaborated. Solvent polarity was shown to exert a profound influence on the yield and composition of ozonides. The influence of fatty acid structure on ozonide formation and the thin-layer chromatography (TLC) of ozonides of a number of different types of unsaturated fatty acids are illustrated. Simple methods for the ozonization of unsaturated fatty acid esters and the cleavage of ozonides by reduction and pyrolysis are described. Applications of ozonolysis to the determination of the structures of geometric isomers, hydroxy, epoxy and cyclic olefinic fatty acids, and the localization of radioactivity along the chain of unsaturated fatty acids are illustrated.

Introduction

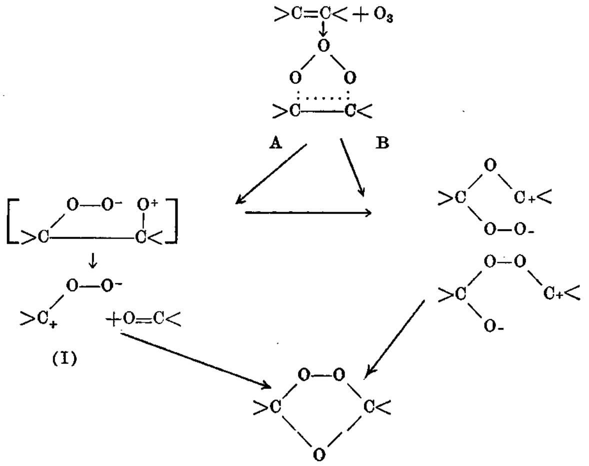
Although the localization of double bonds in unsaturated fatty acids has been made via ozonolysis for many years, the methods have received considerable criticism (1–3). The many different procedures, as well as the wide usage of improved permanganate methods (4,5), attest well to the fact that ozonolysis methods have not been completely satisfactory.

Ozonolysis methods consist of ozonization of the double bonds followed by cleavage of the resulting ozonides into fragments. The positions of the double bonds are deduced from the chain length of the fragments. The purpose of this report is to discuss various factors which are important for the successful application of ozonolysis methods, and to elaborate new methodology. The ozonization and the successive steps of cleavage and analysis may be carried out virtually simultaneously, but since each represents well-defined reactions they will be discussed separately.

Ozonization

The formation of ozonides of unsaturated fatty acid esters in nonparticipating solvents may be represented by the reactions in Scheme I.

Pathway A above is generally referred to as the Criegee mechanism (6) and (I) the Criegee zwitterion. There is irrefutable evidence for this mechanism of ozonide formation. There is also considerable evidence (7–11) for an alternate pathway which does not require a complete breakdown of the molecule. Pathway B represents the type of mechanism that has been proposed for this reaction, but complete agreement on a mechanism has not been attained as indicated by the number of pathways that have been suggested.

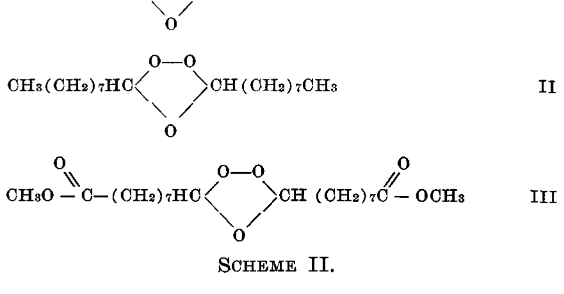


Scheme I.

In the Criegee mechanism (pathway A) the process involves a migration of carbon and its bonding electrons to positive oxygen which results in a fission of the molecule. A migration of carbon and its electrons to positive oxygen is also indicated in the alternate mechanism, pathway B, but apparently other electron shifts occur in concert which keep the molecule from dissociating.

Ozonizations may also be carried out in solvents which participate in the reaction (12–14). These solvents do not yield ozonides as a major product and are not generally employed for the determination of the structure of unsaturated fatty acids, however. A method based on the use of methanol as a participating solvent has been described by Ackman et al. (14). Ozonization of double bonds in methanol gives methoxy hydroperoxides as the major product of the reaction (12). Good results have been obtained with this method on monoenes; its application to polyenes has not been studied.

In accordance with the original Criegree mechanism (Scheme A), complete breakdown in the molecule gives mixtures of both zwitterions and aldehydes and recombinations of these can take place in a number of ways to give a mixture of ozonides. For example, methyl oleate yields three different ozonides, each of which may exist in *cis* and *trans* configuration (7,11,15), as shown in Scheme II.



Scheme II.

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TABLE I

Products of the Ozonization of Methyl Oleate in Mixtures of Pentane and Methylene Chloride

	Pentane	90/10 Pentane/ CH_2Cl_2	75/25 Pentane/ CH_2Cl_2	50/50 Pentane/ CH_2Cl_2	CH_2Cl_2
Parent ozonide	72.1	31.9	20.3	12.4	9.8
Mixed ozonides[a]	10.2	46.4	36.2	19.2	12.9
Aldehydes[b]	4.8	4.3	15.5	19.4	17.3
Other products[c]	12.9	17.4	28.0	49.0	60.0

[a] Mixed ozonides consist of the diester and hydrocarbon ozonides.
[b] Consist of nonanal and methyl azelaaldehydate.
[c] Unidentified highly oxygenated products.

For convenience, I is referred to here as the ozonide of the parent molecule, II as the hydrocarbon ozonide and III as the diester ozonide. The relative amounts of the different possible ozonides, as well as the total yield of all ozonides, are related to the polarity of the solvent in which the reaction is conducted (7). Low yield of total ozonide which occurs in nonparticipating solvents of high polarity is generally accompanied by the tendency toward the formation of equal proportions of all possible mixtures of ozonides of different types and, conversely, the high yields of total ozonide obtained in solvents of low polarity, such as pentane, yield mostly ozonides of the parent molecule. This factor is important in analytical methods which call for the isolation or purification of ozonides. The influence of polarity on the yield of ozonide may be demonstrated by the results obtained with mixtures of pentane and methylene chloride as shown in Table I. The results in Table I show the effects described above. The increase in solvent polarity was attained by increasing the proportion of methylene chloride in the mixture. The influence of polarity may be explained on the basis that solvents of high polarity favor dissociation (pathway A, above) and stability of the zwitterions. The greater stability conferred on the zwitterions by the more polar solvents permits them to interreact to give higher yields of a mixture of ozonides and to react in a variety of ways to yield products other than simple ozonides. Solvents of low polarity do not generally favor dissociation; thus a pathway of the type illustrated by B above predominates giving high yields of total ozonide, as well as mostly parent ozonides.

High yields of parent ozonides can be obtained with fatty acid esters containing up to four double bonds by conducting the reaction in pentane as illustrated by TLC in Figure 1. High yields of total ozonides are also obtained with pentaenes and hexaenes, but the yield of parent ozonide is low (Fig. 1). Apparently, even in pentane considerable dissociation occurs with these fatty esters with the production of a large number of ozonides of different types. That ozonides are the major product of the reaction was determined by infrared spectral analyses (Fig. 2).

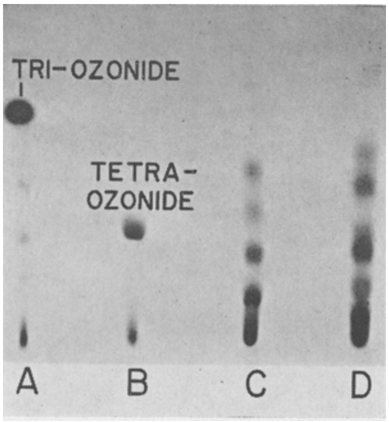


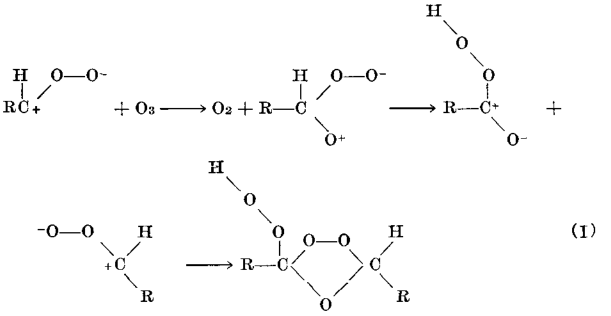
Fig. 1. TLC of the products of ozonization of: (A) methyl linolenate, (B) methyl arachidonate, (C) methyl eicosapentaenoate and (D) methyl docosahexaenoate. Adsorbent, Silica Gel G; solvent, 15% ethyl ether in petroleum ether.

Secondary reactions, or nonselective oxidations, on the ozonization of fatty acid esters are characterized by a strong absorption band at 2.9 μ (—OH stretching region), as well as bands in the carbonyl region of the spectrum as illustrated in Figure 2 by the analysis of the products of the ozonization of methyl oleate in ethyl acetate. The band at 2.9 μ is believed to be due to hydroperoxide or peracid formation, and bands in the carbonyl region indicate the formation of aldehydes and acids. Examination of the infrared spectra of the products of the ozonization of methyl docosahexaenoate and eicosapentaenoate showed no evidence of the formation of such compounds. Thus, although a mixture of products were detected by TLC on the ozonization of these polyunsaturated esters (Fig. 1) they apparently were all ozonides. The expected high concentrations of ozonide groups was verified by the very strong absorption bands at 8.9 to 9.0 μ.

Since nonparticipating solvents may vary widely in polarity, the yield of total ozonides varies considerably in these solvents. Whereas near quantitative yields of ozonides may be obtained in purified pentane (7,16), the yield of ozonides in ethyl acetate or methylene chloride, both of which are nonparticipating solvents, is generally less than 25%. As indication of the mixture of products that may be obtained in the ozonization of unsaturated fatty acid esters in several polar solvents is illustrated with methyl oleate by TLC in Figure 3. The analysis of the products of the ozonization of methyl oleate in methyl alcohol, which is a "reaction participating" solvent as indicated above, is also shown to illustrate that a mixture of products, including some ozonide, is also produced in this solvent.

Criegee (6) has pointed out that the zwitterion in Scheme A above may react in a variety of ways, in addition to reaction with aldehydes to form ozonides. These reactions and the products thereof are ill-defined, but it is apparent that it is these reactions which have been the source of problems with ozonolysis methods. Privett and Nickell (7) showed that the major products of these reactions with methyl esters of fatty acids consist of highly oxygenated compounds with strong hydroxyl absorption. Since ozonides are fairly stable against further attack by ozone, it was proposed (7) that the intermediate zwitterions react further with ozone to yield hydroperoxides.

The method of ozonization is also important for high yields of ozonides (16). When ozonization is carried out by bubbling oxygen containing ozone through a solution of the sample, as commonly employed (17–21), some secondary reactions can be detected by hydroxyl absorption in the infrared spectrum of the products of the reaction (16). It is suspected that because of the speed of the reaction of ozone with double bonds, deficiencies of ozone occur for instants during which the unreacted zwitterions may react with freshly introduced ozone in competition with unsaturated linkages. Ozone reacts very commonly by transfer of an atom of oxygen and release of molecular ozygen (9); that it reacts with the



Scheme III.

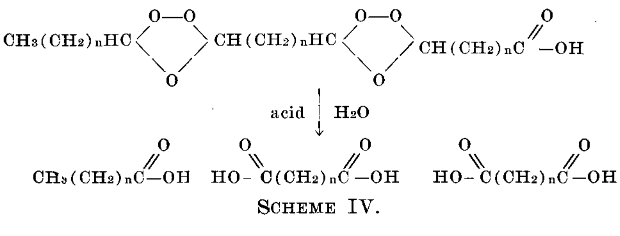
Criegee zwitterion is plausible. Example of a reaction which could lead to a highly oxygenated product is shown in Scheme III.

Zwitterion (I) in Scheme III also may be stabilized by preacid formation. Evidence for reactions of the above type is based on the observation of acid, hydroperoxide, as well as ozonide groups among the highly oxygen compounds formed during the ozonization of fatty acid esters (16).

The highest yields of ozonides are obtained when the sample is dissolved in pentane and introduced into a standard solution of ozone containing an excess over that required for complete reaction with all the double bonds. A simple procedure for the ozonization of unsaturated fatty acids employed in this laboratory is as follows: A standard pentane solution of ozone of about 0.03 M concentration is prepared by bubbling dry oxygen containing 1–3% ozone through 10–20 ml of purified pentane at −65 to −70C until it takes on a distinct blue coloration. The oxygen containing the ozone emerging from the ozone generator should be cooled to about −65C before it is bubbled into the pentane by passing it through a dry ice-acetone trap. Twenty milliliters of this solution is sufficient to ozonize 100 mg of methyl oleate, or a corresponding smaller amount of polyunsaturated fatty acid ester. The sample is dissolved in purified pentane and cooled to a temperature just above that which crystallization occurs, or to −65C. The solution of the sample is added to the standard ozone solution, or a portion thereof if the sample is small, and after a minute or two the excess ozone is removed by bubbling nitrogen through the solution. The solvent is then evaporated to the appropriate volume under reduced pressure for cleavage.

Cleavage of Ozonides

A host of reagents have been employed for the cleavage of ozonides (12). These may be divided into two general groups, depending on whether they involve hydrolytic or reductive reactions. Hydrolytic cleavage generally gives acids, reductive cleavage generally yields aldehydes, but alcohols are produced with some reducing agents. DNPH derivatives may be formed directly by the reaction of ozonides with 2,4-dinitrophenylhydrazine (22–24). Pyrolysis of ozonides may also be controlled to yield aldehydes (25), and this reaction has been made the basis of a simple procedure for the determination of the structure of unsaturated fatty acids by the authors (*vide infra*).



Scheme IV.

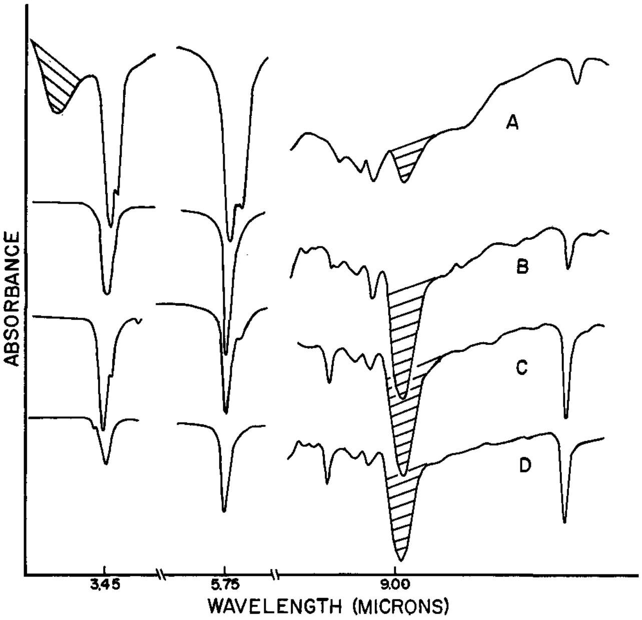


Fig. 2. Infrared spectra of the products of the ozonization of: (A) methyl oleate in ethyl acetate, (B) methyl arachidonate in pentane, (C) methyl eicosapentaenoate in pentane and (D) methyl docosahexaenoate in pentane.

Hydrolytic Cleavage

This type of cleavage may be illustrated by the reaction in Scheme IV.

The reactions in Scheme IV show that in hydrolytic cleavage, dibasic acids are obtained from the proximal end of the molecule and the carbon atoms between the double bonds. The terminal end of the molecule yields

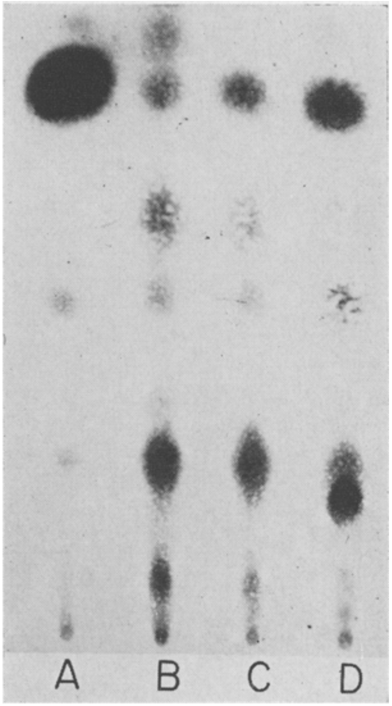
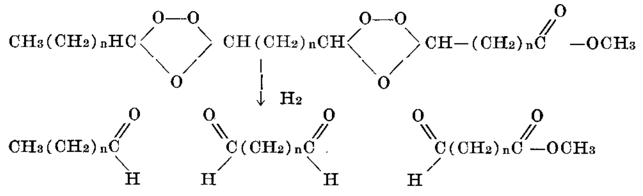


Fig. 3. TLC of the ozonization of methyl oleate in various solvents: (A) pentane, (B) methylene chloride, (C) ethyl acetate and (D) methyl alcohol.



SCHEME V.

a monobasic acid. Acid fragments are usually converted to esters for analysis by GLC. The low molecular weight acids have considerable solubility in aqueous solvents and are difficult to recover; because of their high volatility, they must be converted to butyl (26,27) or higher methyl esters for GLC analysis. The long chain monobasic and dibasic acid are best measured as methyl esters. Thus the analysis of complex mixtures of long and short chain acid fragments, as one frequently encounters in a structural analysis of polyunsaturated fatty acids, is complex and time-consuming. Detailed procedures for the analysis of these compounds have been described by Tullock and Craig (26), Jones and Davison (27) and Stoffel (19).

In addition to the general complexity of the analysis of mixtures of acids obtained by hydrolytic cleavage, ambiguities may be encountered in the interpretation of the results since dibasic acids are products of both the carbon atoms between the internal double bonds as well as the proximal end of the molecule. Moreover, mixtures of acids, especially if not recognized, would cause further complications in the interpretation of the results. There are several ways to get around these problems but they increase the number of manipulations and so make these methods still more complex. There may be occasions or compounds for which a hydrolytic cleavage method is mandatory or most desirable. In this case, the preferred procedure is that described by Stoffel (19) which is based on the well-known method of Klenk and Bongard (20).

Reductive Cleavage

Cleavage by these methods may yield alcohols or aldehydes, depending upon the reagent which is used. The most common methods give aldehydes as illustrated by the reaction in Scheme V.

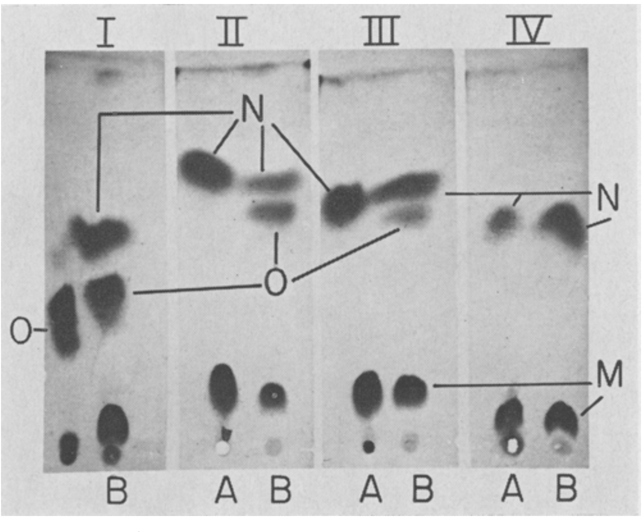


FIG. 4. TLC's of the products of the reduction of the ozonides of methyl oleate, (A) via catalytic reduction with the Lindlar catalyst (30 min reaction time), (B) triphenylphosphine in a methylene chloride. I—10 min. II—100 min. III—180 min. IV—18 hr. N = nonanal. M = methyl azelaaldehydate. O = ozonide. Adsorbent—Silica Gel G; solvent—5% diethyl ether in petroleum ether.

The reaction in Scheme V shows that each section of the molecule of the fatty acid gives a different type of aldehyde. The terminal end of the molecule gives a simple aldehyde, the carbon atoms between the double bonds give a dialdehyde and the proximal end of the molecule gives an aldester. Quantitative reduction of ozonides to aldehydes may be effected by catalytic reduction with the Lindlar catalyst (16).

Triphenylphosphine has also been employed for the reduction of ozonides in several methods (19,28). In the method for the determination of the structure of unsaturated fatty acids described by Stein and Nicolaides (28), the ozonization is carried out in methylene chloride or methyl caprylate. After completion of the ozonization and removal of the excess ozone, triphenylphosphine is added to the solution and after the solution rises to room temperature it is analyzed directly by GLC. Triphenylphosphine itself does not interfere with the GLC analysis as it has a considerably longer retention time than most aldehydes or aldesters. Application of the method to a number of model methyl esters gave the expected fragments in each case (28). However, the reduction of ozonides is not instantaneous under the conditions described in this method as illustrated by TLC (Fig. 4). Even after 3 hr, appreciable amounts of ozonides could be detected (Fig. 4). Thus, in this method the ozonides are apparently cleaved by pyrolysis or by a combination of pyrolysis and reduction. The reduction would also be speeded up at the high temperatures in the injection port and the flash evaporator of the gas chromatograph. Which of these reactions predominates and the composition of the products of the reaction will depend on the temperature and geometry of the flash evaporator, and also the structure of the ozonides. Since the main products of the pyrolysis of the ozonides of monoenes are aldehydes, no severe complications should result on the applications of the method to these compounds. However, a greater abundance of artifacts are generally produced from the pyrolysis of the ozonides of polyenes (*vide infra*). Thus if this method is to be used, sufficient time should be allowed for complete reduction of the ozonides. The extent of the reduction can be readily determined by TLC as illustrated in Figure 4.

Artifacts were eliminated in the use of triphenylphosphine in the method described by Stoffel (19) because, in addition to using a longer reaction time, the aldehydes were isolated and analyzed as their 2,4-dinitrophenylhydrazine derivatives.

A simple method for the catalytic reduction of ozonides which gives essentially quantitative yields of aldehydes has been described by the authors (16). One of the biggest problems in the reduction of ozonides is the quantitative recovery and analysis of short chain aldehyde fragments, especially in the analysis of very small samples. If the reaction is carried out in a volatile solvent, short chain aldehyde fragments will be lost on removal of the solvent, or they may escape detection on GLC analysis if the solvent is not removed. Thus the short chain aldehydes are best analyzed in a solvent which has a relatively long retention time; the limiting factor, in this case, is the concentration of the sample in the solvent, for it is impractical to carry out a reduction on less than 100 μl of solution. Should both long and short chain aldehyde fragments be expected, two analyses are generally performed, one in methylene chloride and the other in a short chain methyl ester (29), dimethyl phthalate (16), or a solvent with a relatively long retention time on GLC. In order to increase the sensitivity of the method to an ultramicroscale, Privett et al. (29) developed a low temperature distillation technique in which the aldehydic fragments were concentrated prior to GLC analysis.

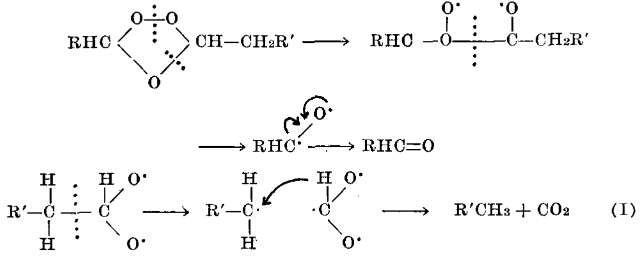
Although aldehydes are generally analyzed by GLC, there may be special cases, as for example in the measurement of specific activity of labeled fragments (19), in which it is desirable to analyze these compounds in the form of their DNPH derivatives. Moreover, the elegant reversed-phase TLC techniques for the analysis of DNPH derivatives developed by Urbach (30) may have special value for the analyses of fragments from fatty acids of unusual structures in this method.

Aldehydes and aldesters may be analyzed very efficiently by GLC with either or both polar and nonpolar phases and temperature programming (25,31). Generally, a complete analysis can be obtained with a silicone column; a second analysis with a polyester column is usually only performed when there is some suspicion of overlapping of aldehyde and aldester peaks or there is a question of the identification of the peaks. With a silicone column the aldesters have retention times which coincide closely with aldehydes having three more carbon atoms in the chain. The retention time of the aldesters relative to aldehydes is much greater on a polyester column than on a silicone column. Thus, a second analysis with a polyester column will generally permit the resolution of components of a mixture of these compounds which have similar retention times on a silicone column.

Pyrolytic Cleavage

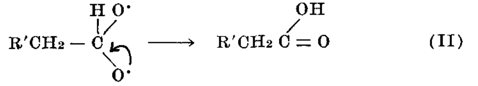
The thermal decomposition of fatty acid ester ozonides in the presence of a hydrogenation catalyst, such as the Lindlar catalyst (32) or palladium on charcoal, also gives quantitative yields of aldehydes, and the reaction has been used as the basis of a microanalytical method for the determination of the structure of unsaturated fatty acid esters (25).

Normally, pyrolysis of fatty acid ester ozonides yields a mixture of acids, hydrocarbons, short chain esters and aldehydes (33–36). The formation of these compounds may be illustrated by the following reactions:



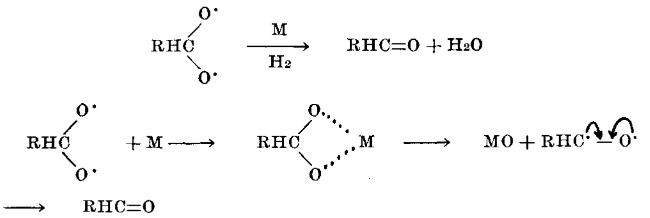
SCHEME VI.

Where R′ arises from the proximal end of the molecule an ester is obtained, the terminal end of the molecule gives a hydrocarbon (I).



$$R'CH_2-\overset{H}{C}(O^{\cdot})O^{\cdot} \longrightarrow R'CH_2C(OH)=O \qquad \text{(II)}$$

Likewise, either mono- or dibasic acids could be produced from reaction (II). In addition to the above reactions, intermolecular reactions could also occur.



M = a hydrogenation catalyst.

SCHEME VII.

The function of the catalyst in the method described by the authors (25), which is used in about a ten-fold excess, may be to effect a simple reduction of the ozonide or the dioxygen radical by virtue of adsorbed hydrogen on its surface, or to react directly with the dioxygen radical. The two reactions may be represented as shown in scheme VII.

Figure 5 shows the GLC analysis of the products of the pyrolysis of the ozonide of methyl linolenate and oleate in the presence and absence of the Lindlar catalyst. In addition to the expected aldehydic fragments, pyrolysis in the absence of the hydrogenation catalyst produced a number of artifacts. The askewed peak in the analysis of methyl linolenate (Fig. 5) probably is due to an acid. Another artifact which arises in the pyrolysis of the ozonides of both oleate and linolenate (Fig. 5) is methyl octanoate. It is believed to arise by elimination of CO_2 (reaction I) from the radical which normally gives methyl azelaaldehydate, according to the reaction shown above. The same compound is produced on the pyrolysis of the ozonide of methyl linoleate. In the pyrolysis of the ozonide of methyl oleate, octane was also produced, apparently by the same reaction (I). When the pyrolysis was carried out in the presence of the hydrogenation catalyst, the results in Figure 5 show that artifact formation was eliminated.

Davision and Dutton (37) have also reported a method for the determination of the structure of unsaturated fatty acids via the thermal decomposition of ozonides. In their method, acids and other products

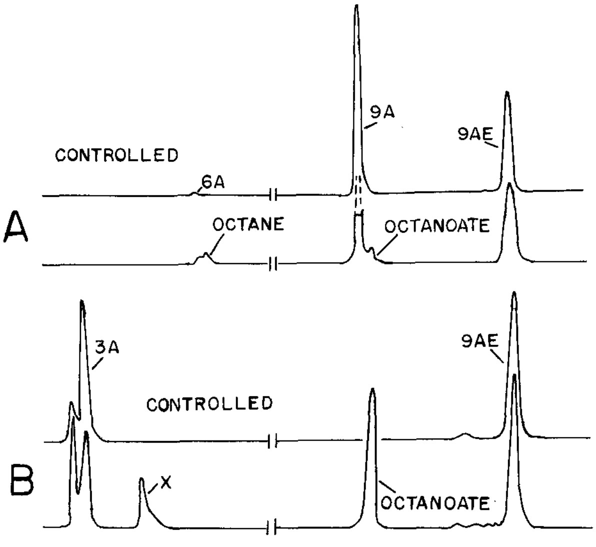


FIG. 5. GLC of the products of the pyrolysis of the ozonides of (A) methyl oleate and (B) methyl linolenate in the absence (uncontrolled) and presence of (controlled) the Lindlar catalyst. Conditions: 30% silicone phase; temperature programmed from 60 to 190C.

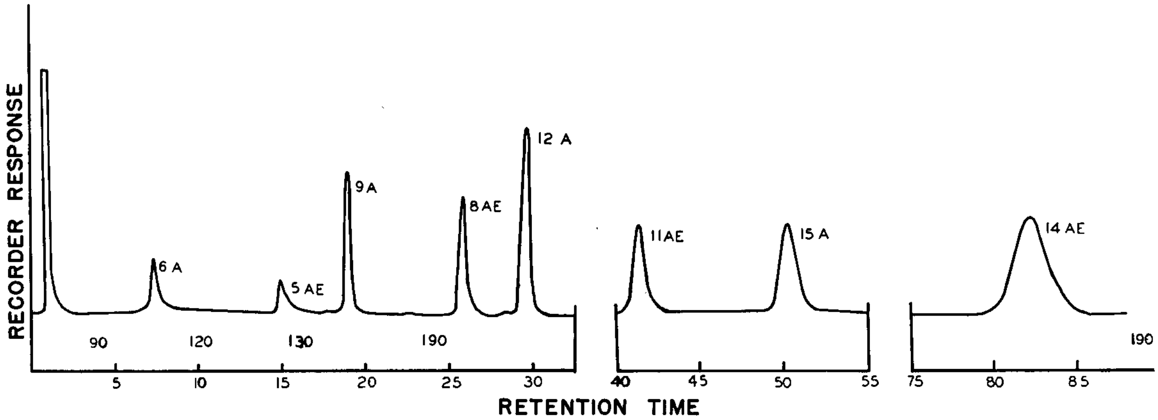


FIG. 6. GLC of the fragments given by reductive ozonolysis of the monoenoates isolated from partially reduced methyl arachidonate. AE = aldester. A = aldehyde. The number = the chain length of these fractions in the shorthand designations of the peaks in the chromatogram. For example, 6A = hexanal. Conditions; 30% silicone phase; temperature programmed from 60 to 190C.

in addition to aldehydes are formed since an oxygen acceptor, such as a hydrogenation catalyst, is not used in the reaction. The acids are removed by reaction with zinc oxide. Whereas the pyrolysis of the ozonides of methyl oleate, and presumably monoenes in general, give high yields of aldehydes, the formation of acids and other products increases greatly with the pyrolysis of polyozonides as illustrated by a comparison of the pyrolysis of the ozonides of oleate and linolenate in Figure 5. Thus, although the acids may be separated in the course of the chromatography of aldehyde fragments, the production of large amounts of secondary reaction products is basically undesirable and could lead to erroneous results.

The pyrolysis of ozonides in the presence of an oxygen acceptor is very simple and may be carried out in the injection port of the GLC instrument in which the subsequent analysis is to be performed. A simple procedure employed in this laboratory (25) for a structural analysis via pyrolysis is as follows: The solution, after ozonization of the sample in the form of methyl ester, is reduced in volume to about 100 μl or less, depending on how much sample is ozonized. An aliquot which contains approximately 10 μg of each aldehyde fragment is introduced by means of a syringe into a small glass tube (25×2 mm I.D.) coated with about 3 mg of Lindlar or other hydrogenation catalyst. The tube is coated by dipping it in a methylene chloride slurry of the catalyst containing 5% calcium sulfate which serves as a binder. After the sample is introduced into the tube the solvent is evaporated in a stream of argon or helium. Then the tube, which is purged of all air by this means, is placed into the top of the column of the GLC instrument opposite the input heaters at a temperature of 200–225C. The carrier gas is turned off for the instant that it takes to insert the glass tube in the column to prevent blow back of the sample and loosening of the column packing. After the analysis is completed, the tube is removed by means of a pair of tweezers. Other techniques, of course, may be employed with different equipment whereby the pyrolysis may be effected in the injection port or flash evaporator of the GLC instrument so as to permit a simultaneous analysis of the fragments.

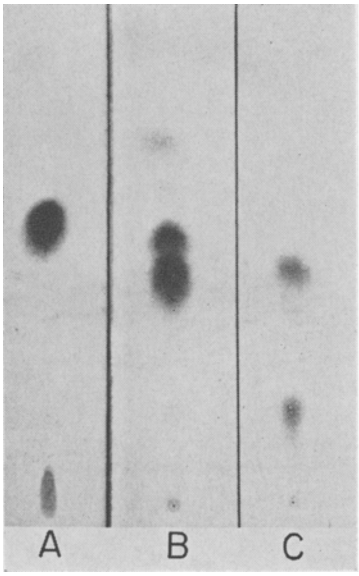


FIG. 7. Thin-layer chromatoplate of the analysis of: (A) methyl ricinoleate, (B), oznoides of methyl ricinoleate (upper spot-*trans* isomer, lower spot-*cis* isomer), (C) products of catalytic reduction of the ozonides. Upper spot-3-hydroxyl nonanol; lower spot-methyl azelaaldehydate. Adsorbent, Silica Gel G; solvent, 1:1 diethyl ether-low boiling petroleum ether.

The advantage of the pyrolysis method is that it is fast and simple. It may be carried out on just a few micrograms of sample in many cases, and the problems associated with the use of a solvent are eliminated. Moreover, since there is no loss of fragments, and the conversion to aldehydes is quantitative, the method may be applied to the quantitative analysis of mixtures of fatty acids (38).

Special Applications

Determination of the Specific Positions of cis and trans Double Bonds and Internal Double Bonds in Polyenes

Generally, the structure of a fatty acid is deduced from the fragments obtained from only the proximal and terminal ends of the molecule. For polyenes it is generally assumed that the double bonds are methylene interrupted; a confirmatory analysis may be made by alkali-isomerization. The determination of the specific positions of the internal double bonds may be made via the partial hydroxylation-permanganate method described by Gunstone and Sykes (39) or de Jong (40), but these methods are long and involved procedures. Isomer composition has been determined in investigations of catalytic and chemical hydrogenation by a combination of fractionation techniques in conjunction with the permanganate-periodate method of structural analysis (26,27). In these procedures selective argentation (41,42) has been employed in various ramifications to separate the geometric isomers. Recently, similar procedures have been employed in an investigation of the products of the selenium or nitrogen oxide isomerization of methyl linolenate (43), the determination of the structure of *trans*-3, *cis*-9, *cis*-12, *cis*-15-octadecatetraenoic acid by Hopkins and Chisholm (44) and the mechanism of the hydrazine reduction of eleostearic acid by Mikolajczak and Bagby (45).

Recently the authors (31) reported a method based on a similar principle for the determination of the specific positions of *cis* and *trans* double bonds, as well as the positions of the internal double bonds in polyenes via ozonolysis. The method involves three steps: 1) partial reduction of the sample with hydrazine so as to give one monoene for each double bond in the polyene; 2) isolation of the *cis* and *trans* monoenes via silver nitrate-TLC; and 3) determination of the structure of the isolated monoenes by ozonolysis using either reduction or pyrolysis techniques as described above. The application of the method (Fig. 6) shows the GLC analysis of the fragments obtained from reductive ozonolysis of the monoenoates isolated from the products of partially reduced methyl arachidonate. This compound should give four *cis* eicosenoate positional isomers, that is, one each with double bonds corresponding to the four positions of the double bonds in the original ester. Examination of the results in Figure 6 show indeed that four eicosenoate isomers with double bonds in the 5,8,11,14 positions, respectively, made up the products of this fraction.

Determination of the Specific Positions of Radioactive Carbon Atoms in C^{14} Tagged Fatty Acids

Generally the radioactivity of specific carbon atoms in the chain of fatty acids is determined by a stepwise degradation of the fatty acids by the Dauben method (46), or a modification thereof, followed by a determination of radioactivity in the fragments. Such methods leave much to be desired because of the low yields of the primary reaction products and the general increase in side reactions as the method is extended along the chain. It is evident that by the determination of the radioactivity of the fragments obtained by reductive ozonolysis, the radioactive carbon atoms can be localized to various sections of the molecule. Stearns and Quackenbush (47) have developed the method further by carrying out a partial reduction of the fatty acid prior to reductive ozonolysis so as to obtain the widest possible shift of the double bonds. This method, which employs a nickel catalyst, permits the localization of the radioactivity to individual carbon atoms in the central portion of the chain. Recently Showell (48) described the isomerization of double bonds with perchloric acid which gives even wider shifts of double bonds than partial catalytic hydrogenation. Thus, application of this technique in conjunction with the Stearns-Quackenbush method should provide a still more detailed analysis.

Determinationn of the Structures of Complex Fatty Acids

Considerable difficulty has been experienced in the application of structural methods of analysis, particularly permanganate methods, to the analysis of fatty acids with oxygen containing functional groups (49–51). The newest methods of ozonolysis have not been widely applied to fatty acids of unusual structures. The reductive ozonolysis method described above has been applied to methyl ricinoleate (methyl 12-hydroxyoctadeca-9-enoate) and methyl epoxy oleate (methyl 12,13-epoxyoctadeca-9-enoate) in this laboratory (52). The TLC of the products of the ozonization, and subsequent reduction of the ozonides of these compounds (Fig. 7 and 8, respectively), showed that the reactions were uncomplicated.

Methyl sterculate does not yield a normal ozonide, but that of a cyclic olefin (51) ozonized under the conditions described above. The ozonides of cyclic olefins, as well as the diketones produced on reduction, may be recognized by their characteristic infrared spectrum as illustrated by the analysis of the products of the reductive ozonolysis of sterculene in Figure 9.

In general, new advances in ozonolysis techniques have greatly simplified the determination of the

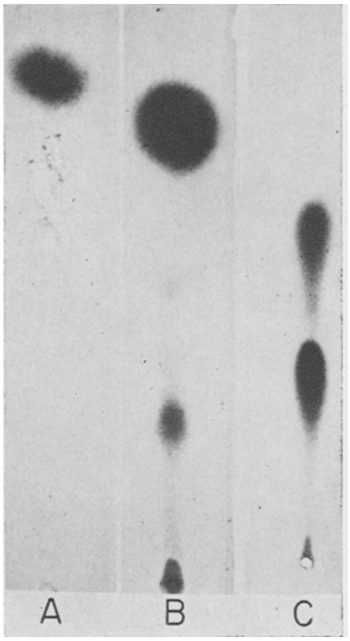


FIG. 8. Thin-layer chromatoplate of the analysis of: (A) methyl 12,13-epoxy-oleate, (B) ozonide of compound (A), (C) products of the reduction of the ozonide. Upper spot = 3,4-epoxy nonanal, lower spot = methyl azelaaldehydate. Adsorbent, Silica Gel G; solvent, 1:1 diethyl ether-low boiling petroleum ether.

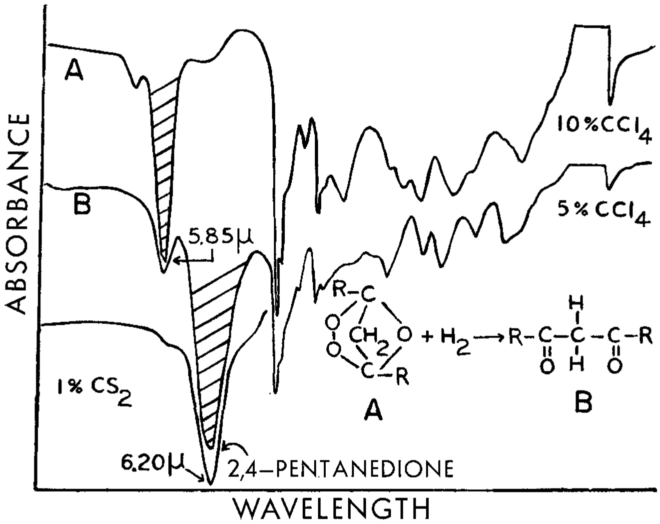


FIG. 9. Infrared spectra of of the products of the reductive ozonolysis of sterculene. (A) Ozonide. (B) Products of the reduction of the ozonide of sterculene.

structure of unsaturated fatty acids. The new methods are rapid, applicable on a microgram scale and may be used for quantitative analysis of mixtures of fatty acids that cannot be analyzed readily otherwise. Methods are available for the determination of the specific positions of *cis* and *trans* double bonds, the internal double bonds of polyenes and the localization of radioactivity in carbon atoms along the chain of C^{14} tagged fatty acids. The new methods also appear to be applicable to a wide variety of fatty acids of unusual structure.

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