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Studies of canine adrenal polyenoic acids: locating double bonds by periodate-permanganate oxidation and gas-liquid chromatography*

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SUMMARY

An improved micromethod has been developed for the location of double bonds in some naturally occurring polyenoic acids by oxidative cleavage of olefinic bonds with a mixture of periodate and permanganate (von Rudloff's oxidant) and analysis of the acidic fragments by gas-liquid chromatography. A method is described for calculation of the double-bond rhythm of polyunsaturated fatty acids. The structures of three polyenoic acids, isolated from canine adrenal lipids by methanolysis and preparative gas-liquid chromatography of the methyl esters, were established as 8,11,14-eicosatrienoic acid, 5,8,11,14-eicosatetraenoic acid, and 7,10,13,16-docosatetraenoic acid.

The classic method for locating the position of olefinic linkages in unsaturated fatty acids consists of an oxidative cleavage of the double bonds with subsequent identification of the resulting fragments. Since the advent of gas-liquid chromatography (GLC), identification of these acidic fragments has become a relatively simple matter, but the problem remains of a dependable procedure for the oxidation of olefins on a microscale. Oxidation by ozonolysis has been used by Stoffel and Ahrens (1, 2), Keppler (3), and Cason and Tavs (4). Although Cason and Tavs claimed that this route resulted in clean-cut cleavage of the double bonds, Benton *et al.* (5) reported that a variety of by-products was formed during the reaction and that interpretations were not necessarily unequivocal. Oxidation by potassium permanganate has also been used widely. James and Webb (6) and Fulco and Mead (7) oxidized polyenoic acids up to C_{20}-tetraenoic acid with potassium permanganate and acetic acid as solvent. Both groups reported that some over-oxidation occurred during the reaction to produce a number of lower mono- and dicarboxylic acids, which were identified by GLC.

A periodate-permanganate oxidation as developed by von Rudloff (16), coupled with GLC analysis of the degradative products, was used by Tulloch (8) for the determination of oleic, linoleic, and linolenic acids in unsaturated oils. His results were in fair agreement with those calculated from the composition of the oils.

For the future investigation of the intermediary metabolism of lipids in this laboratory, the present study was undertaken to establish a more reliable method for the location of double bonds in various polyenoic acids of animal origin. Critical tests were made of various methods for the oxidative cleavage of double bonds by ozonolysis coupled with hydrogen peroxide oxidation of the intermediate products and by periodate-permanganate oxidation. High-purity methyl esters of various monoenoic and polyenoic acids were used as standards for comparisons of the methods.

Details are given of a suitable procedure for the oxidation of some polyenoic acids on a microscale and GLC analysis of the final products. The method has been applied to a study of several polyenoic acids

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obtained from canine adrenal glands. It is of particular interest that the structures of three adrenal polyenoic acids have been established as 8,11,14-eicosatrienoic acid, 5,8,11,14-eicosatetraenoic acid, and 7,10,13,16-docosatetraenoic acid. The presence of the latter polyenoic acid confirms the tentative identification of a C_{22}-tetraenoic acid made by Dailey *et al.* (9) with lipids derived from adrenal tissue. The location of double bonds in this acid agrees with results of a recent study by Klenk and Eberhagen (10) of a C_{22}-tetraenoic acid obtained from the phosphatides of bovine adrenal tissue.

TABLE 1. FATTY ACID COMPOSITION OF TOTAL LIPIDS FROM CANINE ADRENAL TISSUE

Fatty Acid	Peak Number*	Area Per Cent
14:0	1	2
16:0	3	20
16:1	4	3
18:0	8	12
18:1	9	39
18:2	10	10
20:3	11	2
20:4	12	3
22:4	13	3

* See Figure 1.

EXPERIMENTAL METHODS

Materials. All solvents were reagent grade. Fresh adrenal tissue obtained from normal adult dogs was frozen immediately following excision and was stored at −20° for subsequent analysis. Heat-activated silicic acid (Unisil, 100 to 200 mesh, Clarkson Chemical Co., Williamsport, Pennsylvania) was used to purify crude methyl esters obtained by preparative gas chromatography. Reference samples of methyl esters were obtained from the Hormel Institute, Austin, Minnesota. Column packings for analytical gas-liquid chromatography, containing ethylene glycol-succinate and ethylene glycol-adipate polyesters, were obtained from Applied Science Laboratories, State College, Pennsylvania, and Analabs, Hamden, Connecticut. The special packing used for preparative gas chromatography was prepared from Celite, 60 to 80 mesh, and ethylene glycol-adipate polyester. This polyester was prepared by a modification of standard techniques, using a rotating evaporator and methylsulfonic acid as catalyst (11).

Gas-Liquid Chromatography. Microanalyses of methyl esters were made in a Barber-Colman Model 10 instrument (Barber-Colman Co., Rockford, Illinois) equipped with an argon ionization detector containing 56 μc of Ra^{226} as the source of ionizing radiation and in a Chromalab instrument (Glowall Corp., Glenside, Pennsylvania) containing a coiled glass column and equipped with an argon ionization detector similar to that described recently by Lovelock (12) with 22.5 μc of Ra^{226}. Routine identifications were based on comparisons of retention times with those of standards. Results were calculated in terms of uncorrected area per cent. The linearity of each detector was tested with known mixtures of standards (13).

Preparation of Methyl Esters of Fatty Acid from Adrenal Tissue. Four pairs of adrenal glands, 7.1 g wet weight, were extracted by grinding them in sand with four portions of chloroform–methanol 2:1. To the pooled extract (90 ml) was added 18 ml water with vigorous mixing. After standing under refrigeration in the presence of nitrogen for 15 to 18 hours, the chloroform layer containing total adrenal lipids was separated and the solvent was evaporated *in vacuo* at 40°. The yield of crude lipid was about 2.3 g.

Methanolysis of the crude lipid was carried out by the method described by Stoffel *et al.* (14). A total of 2.1 g of methyl esters was obtained by extraction with hexane. A 25% solution of methyl esters, prepared in hexane, was stored under nitrogen at −20°. GLC analysis of the methyl esters on adipate polyester is shown in Figure 1. Identification of the peaks was based on comparisons of the retention times with those of standards. The chain lengths of three polyenoic acids, numbered 11, 12, and 13, were confirmed by GLC analysis of hydrogenated samples. Table 1 shows area per cent of each of the major fatty acids found in the mixture.

Isolation of Polyenoic Acids by Preparative Gas-Liquid Chromatography. A preparative column, 8 ft in length with an internal diameter of $^3/_8$ in., was packed with 30% ethylene glycol-adipate polyester coated on 60 to 80 mesh Celite and installed in a Barber-Colman Model 10 instrument. Collection of appropriate peaks was made by passing the effluent gas stream into a three-way glass joint with capillary restrictors so that about 10% of the gas was diverted to an argon detector while 90% of the gas passed directly through 3 to 4 feet of Teflon capillary tubing ($^1/_{16}$ in. internal diameter), which served as a collector. Temperatures for the column, detector, and flash heater were 210°, 220°, and 250°, respectively. Loads up to about 0.3 ml of 25% methyl esters in hexane could be separated with little reduction in column efficiency; to save time however, 1-ml portions of the hexane solution were used for each injection. The total sample of methyl esters was chromatographed in this way by a

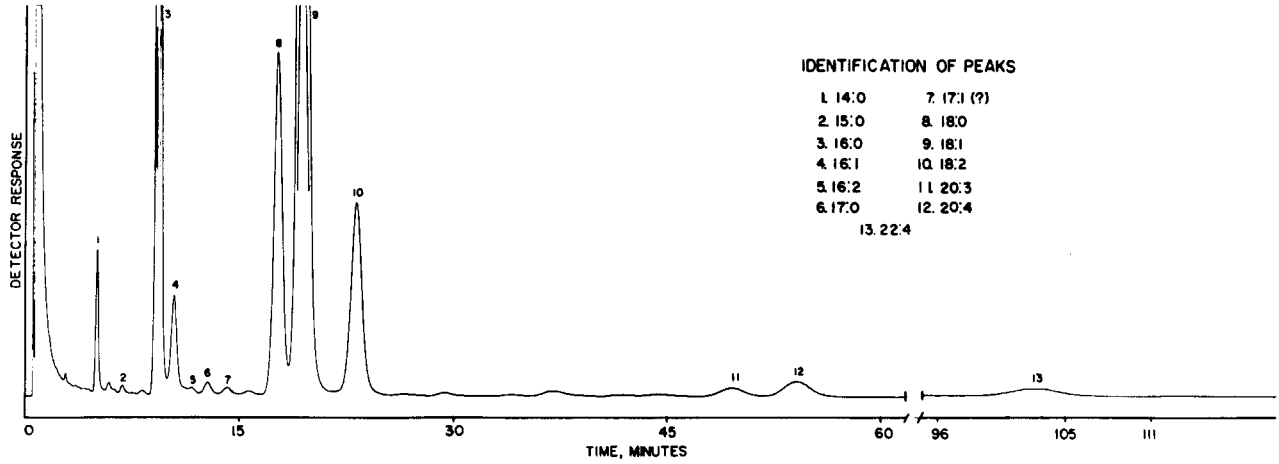

FIG. 1. GLC analysis of methyl esters from canine adrenal tissue. Column (6 ft x 1/8 in.) packed with 15% ethylene glycol-adipate on 80 to 100 mesh Chromosorb W (Johns-Manville Corp., New York, N. Y.); temperature, 200°; argon inlet pressure, 20 psi; sample size, 1 μl of 2% solution in hexane.

series of eight injections. Appropriate samples of the effluent gas stream were carried through the Teflon tubing, and condensation occurred at room temperature without further cooling. For each desired fraction, a new length of Teflon tubing was installed at the time indicated by observing the response of the detector. In order to prevent contamination of the sample by material from adjacent peaks, collection was made only during the middle half of the elution time. After each collection, the sample was rinsed immediately from the capillary tubing with petroleum ether and was stored under nitrogen at −20°.

The collected samples corresponding to peaks 11, 12, and 13 (Fig. 1) contained small percentages of 16:0, 16:1, 18:0, and 18:1 methyl esters. In addition, the sample corresponding to peak 11 was found to be a mixture of 20:3 and 20:4 (1:1 ratio of areas) and it was necessary to rechromatograph this sample; the resultant product was slightly less than 90% pure.

At 210°, elution of the adipate polyester occurred to a small extent, causing a considerable contamination of the products. Silicic acid chromatography was used for purification of the products at this point. The columns (6 in. by 1/2 in.) contained 4 g of Unisil silicic acid, and the methyl esters were eluted with 50 ml of benzene—hexane 1:1, with complete retention of the impurities by the silicic acid.

Analyses of these fractions by GLC are shown in Figure 2. The traces of methyl esters of 16:0, 16:1, 18:0, and 18:1 in fractions 12 and 13 (Fig. 2) were considered negligible for the purposes of this study. If desired, effective purification may be made by repeating the preparative gas-liquid chromatography of the product as shown in the case of fraction 11 (Fig. 2). The final weights of the three samples of polyenoic esters were: fraction 11 (20:3), 1.3 mg; fraction 12 (20:4), 6 mg; fraction 13 (22:4), 8 mg. The samples were dissolved in 1 ml, 2 ml, and 3 ml *t*-butanol, respectively, for oxidation.

Ozonolysis of Reference Esters. Ozonolysis was performed at −17° in several solvents by using the Welsbach Ozonator (The Welsbach Corp., Philadelphia, Pennsylvania). The intermediates were further oxidized to acids with 7% hydrogen peroxide in dilute acetic acid or sodium hydroxide solution. The mixture was incubated at 37° for 3 days after which the solvents were removed by distillation (miniature Vigreaux column at 103°) or lyophilization (0° and 1 mm Hg) under alkaline conditions. Methyl esters were prepared with diazomethane by using the apparatus described by Schlenk and Gellerman (15). Analysis by GLC of the products from oleic, linoleic, and arachidonic acids disclosed a number of mono- and dicarboxylic acids in addition to those predicted on the basis of the structures to the unsaturated fatty acids. These results were in agreement with those obtained by Benton *et al.* (5).

Periodate-Permanganate Oxidation of Reference Esters and Methyl Esters of Three Adrenal Polyenoic Acids. A modification of the oxidative procedure developed by von Rudloff (16) was followed. While von Rudloff used a titrimetric method to determine the microquantity of acidic products, the present procedure involved recovery of the fragments by extraction and identification by GLC. In addition, the quantity of reagents was increased slightly to provide excess oxidant for these highly unsaturated acids. The reaction mixtures consisted of 4 ml of von Rudloff's oxidant containing 0.018 M sodium periodate and 0.0025 M potassium permanganate, 2 ml of 0.1 M

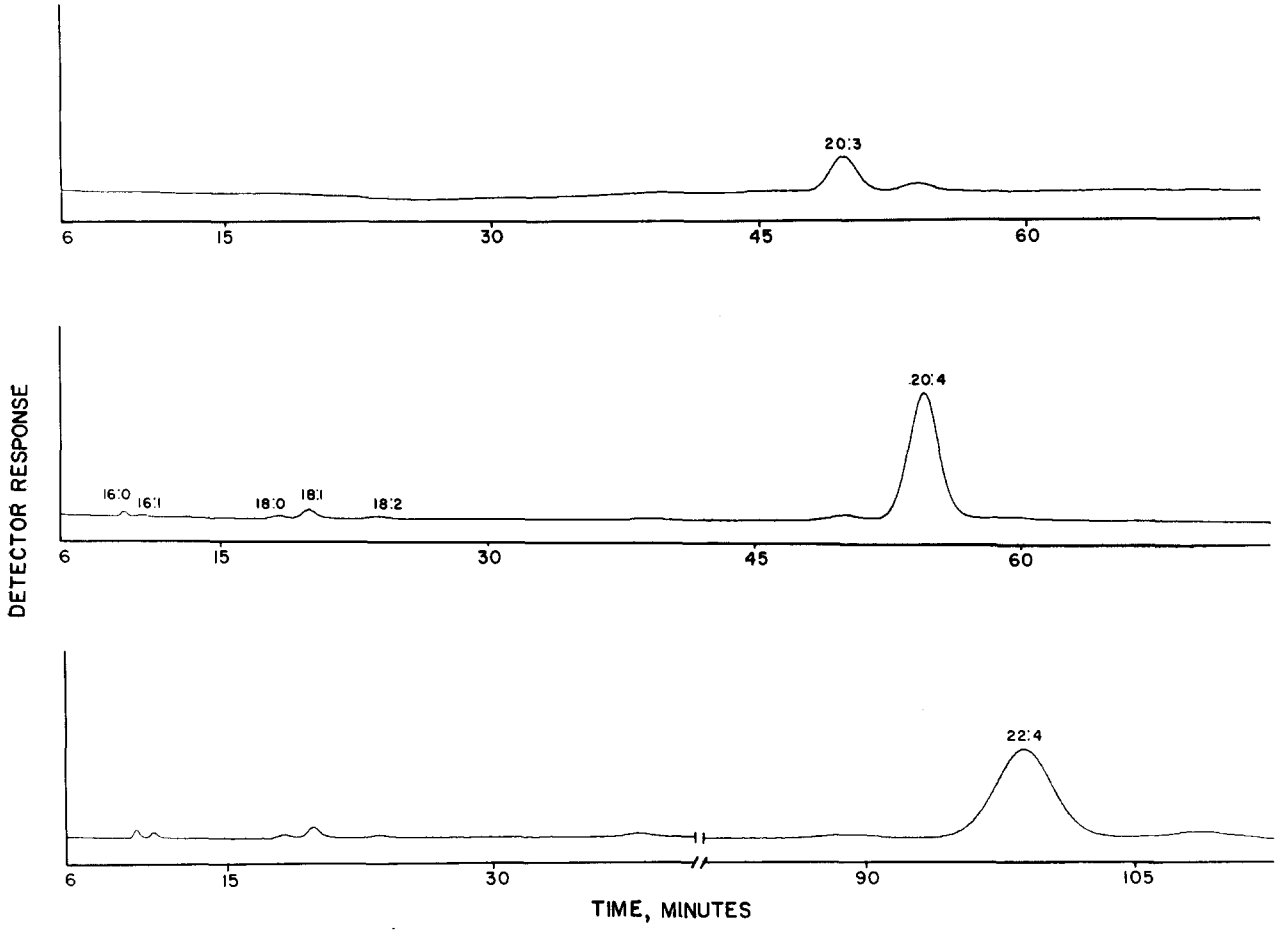

FIG. 2. GLC analysis of purified adrenal polyenoic esters on adipate polyester (see Fig. 1 for conditions).

sodium carbonate, and 3 ml of *t*-butanol. The quantity of polyenoic ester used for the oxidation ranged from 1 to 3 mg. A blank containing 4 ml of *t*-butanol was carried through the entire procedure.

Oxidation was carried out at room temperature (24° to 27°) for 6 hours in a 20-ml glass-stoppered centrifuge tube with constant shaking. The reaction was stopped by the addition of sodium metabisulfite, and the acids were converted to their potassium salts by the addition of a pellet of potassium hydroxide. *t*-Butanol and most of the water were removed *in vacuo* at 50°. The residue was made strongly acidic with 15% sulfuric acid (no more than 1 ml was needed), and the acids were extracted with 4 portions of ether, 2 ml for the first two extractions and 1.5 ml for the last two extractions. The pooled ether extract was dried over anhydrous sodium sulfate and was filtered into a 10-ml graduated centrifuge tube with glass stopper. The tube was placed in a beaker of cold water (ca. 14°), and the ether was gently evaporated with a stream of nitrogen to a final volume of exactly 0.5 ml (some loss of very low boiling acids may occur). Enough dry methanol was added to make a final solution of 10% methanol in ether. Esterification of the acids was carried out in the centrifuge tube with gaseous diazomethane as described by Schlenk and Gellerman (15). Excess diazomethane was carefully removed by nitrogen (at 14°), and the mixture was ready for GLC identification of the fragments.

RESULTS

Studies of the cleavage of olefinic bonds by ozonolysis were not easily interpreted because it was not possible to find a condition under which secondary reaction products were not produced. The choice of solvents appears to have a bearing on this matter, and further studies with a variety of solvents and conditions may lead to the development of a suitable alternate method for microanalysis.

The conditions described by von Rudloff for the oxidation of triglycerides (16) were adapted to the

TABLE 2. STRUCTURES OF REFERENCE POLYENOIC ACIDS AS DETERMINED BY PERIODATE-PERMANGANATE OXIDATION

	Degradation Products			
Fatty Acid	Monocarboxylic Acids	Dicarboxylic Acids	Position of Double Bonds	Approximate Relative Amounts of Isomers
Oleic	Nonanoic	Azelaic	9	
Linoleic	Caproic	Azelaic	9, 12	
		Malonic*		
Linolenic	Propionic	Azelaic	9, 12, 15	
		Malonic*		
Arachidonic	Caproic	Glutaric	5, 8, 11, 14	4
	Propionic	Suberic	8, 11, 14, 17	1
		Malonic*		
Eicosapentaenoic	Propionic	Glutaric	5, 8, 11, 14, 17	
		Malonic*		
Docosahexaenoic	Propionic	Succinic	4, 7, 10, 13, 16, 19	
		Malonic*		

* Malonic acid was never obtained in greater than trade quantities in these studies.

methyl esters of polyenoic acids as he had suggested. On the basis of the results with the standards, the use of a mixture of periodate and permanganate was clearly the method of choice. The results obtained with six reference esters are summarized in Table 2.

The results obtained with oleic, linoleic, and arachidonic acids indicated that the terminal portions of these standards were converted equally well to corresponding mono- and dicarboxylic acids. Qualitatively, propionic acid was easily observed as a fragment from linolenic, eicosapentaenoic, and docosahexaenoic acids by using an adipate polyester at 52°. The yield of propionic acid was routinely low, however, and quantitative estimations were not possible. This observation has been attributed to partial losses of propionic acid during various manipulative steps involving evaporation of solvent. Other low-boiling monocarboxylic acids ($<C_6$) are likely to be lost to some extent during evaporation. For quantitative estimations of mixtures, measurements of the areas of the dicarboxylic acids are likely to be more reliable since these products are considerably less volatile. Alternatively, high-boiling esters may be used in place of methyl esters when propionic acid or other low-boiling components are observed. Tulloch has used phenacyl esters for this purpose (8). More recently, Oette and Ahrens have reported that sufficiently low volatility may be obtained with 2-chloroethyl esters to permit quantitative recovery from solvents (17).

In all of the studies of reference esters, the intermediate portions of the polyenoic esters were apparently further oxidized to carbon dioxide since practically no malonic acid was found. This destruction of intermediate fragments was also observed with linoleic acid by von Rudloff (18). This result is not surprising since Huebner *et al.* have described an abnormal periodate oxidation of β-dicarbonyl compounds (—CO-CH_2CO—) such as malonic acid, which was converted to carbon dioxide and formic acid (19). The periodate oxidation of added free malonic acid was confirmed under the conditions of the present study with von Rudloff's oxidant.

Figures 3A and 3B show the chromatograms of the dicarboxylic and monocarboxylic acids (methyl esters) obtained from three polyenoic acids derived from canine adrenal lipids. The peaks were identified by comparing their retention times with those of standards. In addition, a GLC column containing adipate polyester was maintained at 52° for the determination of C_2 to C_5 monocarboxylic acids; no products within these chain lengths were found in any of the studies.

Several small peaks appeared routinely after oxidation of samples, and the same peaks were observed in blanks. These traces of low-boiling products may result from oxidizable impurities in the *t*-butanol.

A small peak shown in Figure 3A at 3.5 min for 20:4 and 22:4 was due to malonic acid that was not completely oxidized. Three additional minor components, at 15.6, 18.7, and 25.9 min, were attributed to trace contamination by 16:0, 16:1, and 18:0 in the original samples of 20:4 and 22:4 as shown in Figure 2. No such peaks were observed with 20:3, which had been rechromatographed prior to oxidation.

Fraction 11 (20:3) was oxidized to a major C_8 dicarboxylic acid and a minor C_5 dicarboxylic acid (see Fig. 3A). It was believed at first that the sample contained two positional isomers, 8,11,14-eicosatrienoic acid and 5,8,11-eicosatrienoic acid, with the former isomer as the major component. If this were the case, however, two corresponding monocarboxylic acids

(C_6 and C_9) would have been formed. Since only hexanoic acid was observed and since the ratio of areas of the dicarboxylic acids (C_8:C_5, 9 to 1) was similar to that of 20:3 to 20:4 in fraction 11 (Fig. 2), it was reasonable to conclude that the C_5 dicarboxylic acid shown in Figure 3A was produced by oxidation of arachidonic acid contaminating 20:3 in fraction 11. Peak 11 (Fig. 1), therefore, was a C_{20} acid with 3 double bonds, a C_8 saturated carboxyl-terminal segment, and a C_6 saturated methyl-terminal segment. If one assumes the divinylmethane rhythm of double bonds, these data correspond to 8,11,14-eicosatrienoic acid.

Fraction 12 contained one major polyenoic acid with 20 carbon atoms, 4 double bonds, and saturated C_5 (carboxyl) and C_6 (methyl) terminal groups. Peak 12 (Fig. 1) was therefore 5,8,11,14-eicosatetraenoic acid (arachidonic acid).

Similarly, fraction 13 (22:4) consisted of a single positional isomer and, on the basis of oxidation to a C_7 dicarboxylic acid and C_6 monocarboxylic acid, was assigned the structure 7,10,13,16-docosatetraenoic acid.

With regard to the yield of methyl esters from the terminal groups of these polyenoic acids, ratios of di- to monocarboxylic acids were calculated by comparing areas to an internal standard (methyl laurate). The theoretical molar ratio of the terminal products should be 1, but the results (di:mono) were 1.1 for 20:3, and 1.2 for 20:4 and 22:4. The slightly higher ratio obtained in all three cases may reflect a small loss of methyl hexanoate during one or more of the steps involving evaporation of solvent.

DISCUSSION

It has been well established that double bonds present in naturally occurring nonconjugated polyenoic acids are arranged in a divinylmethane rhythm (=$CHCH_2CH$=) (1, 2, 20). By assuming this arrangement of double bonds and having determined the terminal groups by oxidation and GLC analysis, one can readily deduce the molecular structure if either the number of double bonds or the number of carbon atoms is known. Of the last two values, the total number of carbon atoms may be determined easily by hydrogenation of the sample and GLC analysis. By this three-step approach (determination of chain length, determination of terminal groups by periodate-permanganate oxidation, and assumption of the divinylmethane arrangement), the structures of three polyenoic acids from adrenal lipids were derived from extremely small samples of the pure acids.

It must be emphasized that difficult interpretative problems may arise in certain cases with oxidative studies of the type described here. Since malonic acid is destroyed by periodate (19), a polyenoic acid con-

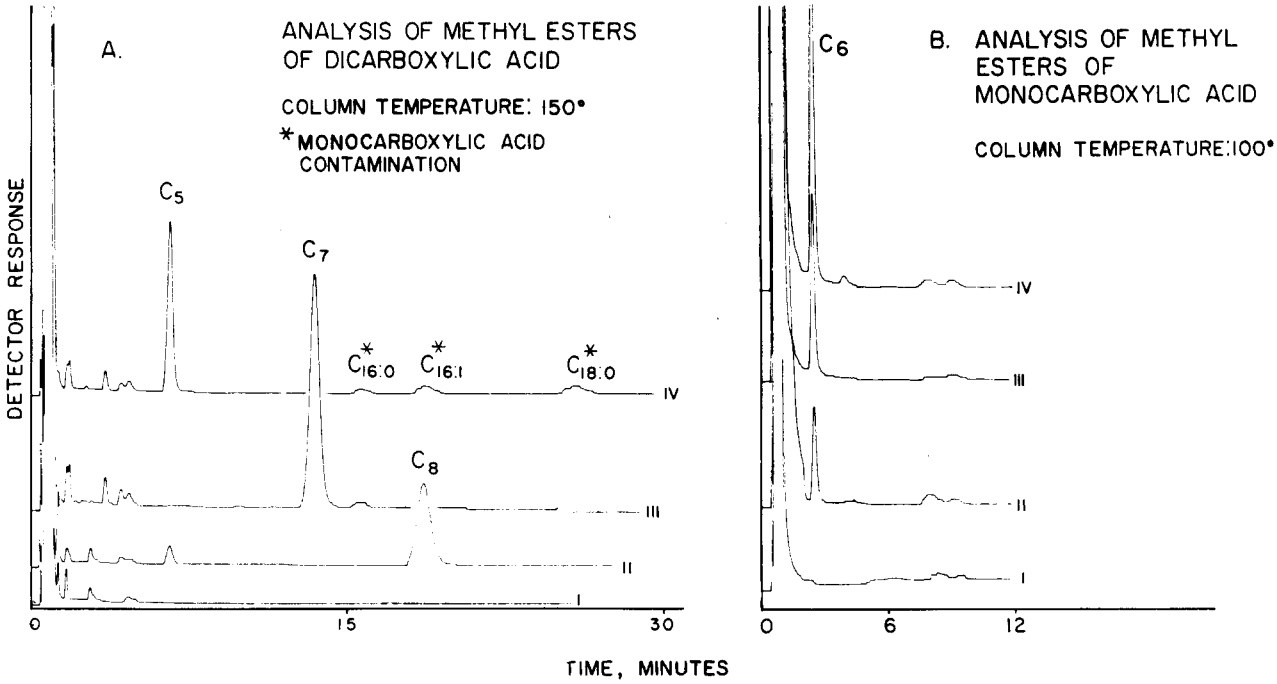

FIG. 3. GLC analysis of acidic products obtained from the oxidation of blank (I), 20:3 (II), 22:4 (III), and 20:4 (IV). Column (6 ft x 1/4 in.) packed with 14% ethylene glycol-succinate on 80 to 100 mesh Chromosorb W.

taining a Δ^3-bond will yield only a monocarboxylic acid on oxidation with periodate-permanganate. For example, 3,6,9,12,15-eicosapentaenoic acid would be cleaved initially to pentanoic acid and 5 molecules of malonic acid, which would be further oxidized to carbon dioxide and formic acid. Nevertheless, the correct structure may be deduced for this acid by locating 5 double bonds, in a divinylmethane rhythm, on the basis of the pentanoic acid, which establishes the position of the first double bond from the methyl-terminal end of the molecule.

Oxalic acid is also destroyed by von Rudloff's oxidant, but in this case permanganate is the oxidant. A similar complication will be observed, therefore, with polyenoic acids containing a Δ^2-bond.

Structures in which the double bonds are randomly arranged cannot be deduced by the three-step method described for the adrenal polyunsaturated fatty acids. For example, 4,8,12,15,17-docosapentaenoic acid would be cleaved to pentanoic acid and a mixture of several dicarboxylic acids among which the carboxyl-terminal fragment could not be distinguished easily.

A simple formula, shown below, may be used for the determination of rhythms of double bonds in some polyenoic acids, provided the total number of double bonds is known, in addition to information obtained from oxidative cleavage and hydrogenation of the sample.

$$\frac{N_t - N_{al} - N_{car}}{N_d - 1} = R$$

where

N_t = total number of carbon atoms in the molecule,
N_{al} = number of carbon atoms in the alkyl-terminal fragment,
N_{car} = number of carbon atoms in the carboxyl-terminal fragment,
N_d = number of double bonds in the molecule.

If

$R = 2$ — completely conjugated system of double bonds,
$= 3$ — divinylmethane rhythm (methylene interruption),
$= 4$ — ethylene interruption,
= noninteger — complex interruption of double bonds.

Example (1) Fraction 12: $N_t = 20$, $N_{al} = 6$, $N_{car} = 5$, $N_d = 4$.

$$\frac{20 - 6 - 5}{4 - 1} = 3\text{(divinylmethane rhythm)}$$

Example (2) 4,8,12,15,18-docosapentaenoic acid: $N_t = 22$, $N_{al} = 4$, $N_{car} = 4$, $N_d = 5$.

$$\frac{22 - 4 - 4}{5 - 1} =$$

3.5(complex interruption of double bonds)

The solution is not by itself unique when $R = 3$ or 4 since hypothetical acids can be constructed for which the result is misleading. For example, 5,9,11,14-eicosatetraenoic acid, an isomer of arachidonic acid with a complex rhythm of double bonds, gives an R of 3. But such cases would be clearly differentiated from a simple divinylmethane rhythm by the fact that oxidation with periodate-permanganate would yield two or more dicarboxylic acids and only one monocarboxylic acid. As discussed previously, structures such as these cannot be deduced correctly without further information.

On the other hand, if $R = 2$, the solution is unique for a fully conjugated system; if R = a noninteger, the solution is unique for a complex rhythm.

With typical naturally occurring polyunsaturated fatty acids, analysis by the formula described will provide further evidence that a given pure sample contains a divinylmethane rhythm of double bonds.

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