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The Determination of the Structure of Unsaturated Fatty Acids on a Micro Scale with the Gas-Liquid Chromatogram

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It has been pointed out in previous publications (James & Martin, 1956; James & Wheatley, 1956) that some information on the structure of unsaturated acids can be obtained from a study of their times of emergence from a gas-liquid chromatogram column. In general, the more double bonds in the molecule the more rapidly the acid moves, mono- and di-unsaturated acids running between the

corresponding saturated acid and its next lower homologue when the column stationary phase is a saturated paraffin hydrocarbon (e.g. Apiezon M vacuum stopcock grease). The position of the double bond also affects the chromatographic behaviour: e.g. methyl-9:10-octadecenoate has a retention volume (James & Martin, 1956) relative to methyl myristate of 4.75, methyl-6:7-octadecenoate 4.87

and methyl-4:5-octadecenoate 4.95. These differences, however, are insufficient to allow separation on any but very long columns, so that precise identification of structure by rate of movement alone is not possible.

Oxidative degradation of unsaturated acids, followed by identification of the mono- and dicarboxylic acids produced, is the classical method of determining structure. Application of this technique on the micro scale necessitates a sensitive technique for separating and identifying the degradation products. The gas-liquid chromatogram not only fulfils this purpose but also serves as a method of preparing the unsaturated acids themselves.

EXPERIMENTAL

The type of column used has been described earlier (James & Martin, 1956), but has been slightly modified in that the support for the stationary phase (Celite 545) is sieved to remove 'fines' (passing a 200-mesh sieve). The stationary phase is melted and mixed with hot graded Celite 545 in the proportion 1:4 (w/w). Columns are packed by vibration as described by James & Martin (1952). In this way 4 ft. columns having efficiencies of up to 3000 theoretical plates for a peak emerging after 2 hr. are produced. The detector used is the gas-density meter (Martin & James, 1956), which measures the difference in density between the stream of gas from the chromatogram and the stream of gas from the reference column.

Collection of samples from the gas chromatogram is carried out by attaching with silicone-rubber tubing a calcium chloride tube loosely packed with defatted cotton wool wetted with methanol. The end of the collection tube fits tightly against the exit tube of the detector so that the vapour emerging does not come into contact with the silicone rubber. When the peak has emerged, the tube is removed and the collected material eluted with three 5 ml. portions of ether. Where peaks overlap only the front or back portions of the zones are collected to prevent

contamination. The ether is then evaporated in the 10 ml. conical centrifuge tube in which the oxidation is carried out.

Oxidation of the collected sample is carried out by dissolving the unsaturated acid in a few drops of acetic acid, adding 100 mg. of powdered KMnO_4 and heating the tube on the water bath at 70° for 1 hr. At the end of this period water (3 ml.) is added together with potassium metabisulphite to remove excess of permanganate and MnO_2 . The solution is made strongly acid with 5N- H_2SO_4 and extracted five times with 5 ml. portions of ether. The ether solution is dried over anhydrous Na_2SO_4 . Esterification of the extracted acids is carried out by treatment with diazomethane prepared essentially as described by Arndt (1943). It is necessary to distil the diazomethane from the reaction mixture in which it is formed, otherwise extra peaks due to side-reaction products will be produced on the chromatogram. The diazomethane and ether are distilled directly into a conical centrifuge tube in which the ethereal solution of the free acids has been evaporated. After standing for 30 min. at 0° the ether and excess of diazomethane are carefully evaporated on the water bath. The residue is dissolved in a few microlitres of a substance (such as methyl pentadecanoate) relatively unlikely to overlap with the expected zones of the chromatogram, and the solution is taken up in a micropipette and applied to the column. The behaviour of the column should be previously standardized with a mixture of methyl esters of known mono- and dicarboxylic acids.

RESULTS AND DISCUSSION

Mono- and di-unsaturated acids

In Fig. 1 is shown a separation of straight-chain monocarboxylic acids from C_5 to C_{14} and dicarboxylic acids from C_4 to C_{11} on the paraffinic stationary phase Apiezon M grease at 197° . The dicarboxylic acid emerges just before the monocarboxylic acid of similar molecular weight. Fig. 2 shows a reversal of order of emergence with a stationary phase of

Fig. 1. Separation of mono- and di-carboxylic acids on a predominantly paraffin stationary phase. Column length 4 ft.; stationary phase Apiezon M vacuum stopcock grease; temperature 197° . Nitrogen pressure 32 cm. Hg above atmospheric pressure, detector at atmospheric pressure. Load 0.6 mg. Peaks in order of appearance: (1) air introduced in loading the sample, (2) methyl *n*-pentanoate, (3) methyl *n*-hexanoate, (4) dimethyl succinate, (5) methyl *n*-heptanoate, (6) dimethyl glutarate, (7) methyl *n*-octanoate, (8) dimethyl adipate, (9) methyl *n*-nonanoate, (10) dimethyl pimelate, (11) methyl *n*-decanoate, (12) dimethyl suberate, (13) methyl *n*-undecanoate, (14) dimethyl azela-ate, (15) methyl *n*-dodecanoate, (16) dimethyl sebacate, (17) methyl *n*-tridecanoate, (18) dimethyl hendecanedioate, (19) methyl *n*-tetradecanoate.

aromatic character (an aromatic extract of a high-boiling lubricating oil). In Table 1 is given a list of retention volumes of these acids relative to methyl myristate on the two types of stationary phase. All the acids are sufficiently well resolved to make identification easy. Fig. 3 shows the relationship between log (time of emergence) and chain length for the two types of acid. The CH_2 -separation factor (James & Martin, 1956) is 1.54 for both types of acid. In the permanganate oxidation of an unsaturated acid, the mono- and di-carboxylic acids produced initially are further attacked by the permanganate to give a homologous series of acids of shorter chain length. A number of other oxidation procedures were tried, including ozonolysis followed by oxidation with hydrogen peroxide, performic acid or silver oxide in alkaline or acid solution, and hydroxylation followed by oxidation with periodate, but in all cases many side products that obscured the main products were formed. The permanganate oxidation, while not ideal, has the advantage that it is easy to pick out the two homologous series of mono- and di-carboxylic acids and to identify the main

products. An oxidation time of 1 hr. was chosen, since an experimental study showed that under these conditions the maximum yield of fission products was obtained with the secondary oxidation products present in quantities diminishing along the homologous series.

It is of course necessary to know the chain length of the unsaturated acid to enable the position of the double bonds to be defined by identification of the fission products. It has been demonstrated that the mono- and di-unsaturated acids move between the corresponding saturated acid and the saturated acid possessing one less carbon atom. In this way the chain length of the unsaturated acids of this group can be determined. The lack of even impure samples of polyunsaturated acids has made it impossible so far to generalize on the chromatographic behaviour of acids possessing more than two double bonds.

With mono-unsaturated acids identification of only one fission product is necessary; with di-unsaturated acids at least two products must be defined; and with more unsaturation all products need to be identified. It has been found possible

Fig. 2. Separation of mono- and di-carboxylic acids on a more polar stationary phase than in Fig. 1. Column length 4 ft.; stationary phase, an aromatic extract of a high-boiling-point lubricating oil; temperature 197°. Nitrogen pressure 11.2 cm. Hg above atmospheric pressure. Peaks in order of appearance: (1) air, (2) methyl *n*-octanoate, (3) dimethyl glutarate, (4) methyl *n*-nonanoate, (5) dimethyl adipate, (6) methyl *n*-decanoate, (7) dimethyl pimelate, (8) dimethyl suberate, (9) methyl *n*-dodecanoate, (10) dimethyl azela-ate, (11) dimethyl sebacate, (12) methyl *n*-tetradecanoate.

Table 1. Retention volumes of methyl esters of mono- and di-carboxylic acids at 197° on two stationary phases (relative to methyl myristate)

Monocarboxylic acid	Stationary phase		Dicarboxylic acid	Stationary phase	
	Apiezon M	Aromatic		Apiezon M	Aromatic
<i>n</i> -Tetradecanoate	1.0	1.0	$\text{HO}_2\text{C}\cdot[\text{CH}_2]_{10}\cdot\text{CO}_2\text{H}$	0.882	—
<i>n</i> -Tridecanoate	0.642	—	$\text{HO}_2\text{C}\cdot[\text{CH}_2]_9\cdot\text{CO}_2\text{H}$	0.57	0.772
<i>n</i> -Dodecanoate	0.415	0.422	$\text{HO}_2\text{C}\cdot[\text{CH}_2]_8\cdot\text{CO}_2\text{H}$	0.378	0.502
<i>n</i> -Undecanoate	0.264	—	$\text{HO}_2\text{C}\cdot[\text{CH}_2]_7\cdot\text{CO}_2\text{H}$	0.228	0.326
<i>n</i> -Decanoate	0.169	0.184	$\text{HO}_2\text{C}\cdot[\text{CH}_2]_6\cdot\text{CO}_2\text{H}$	0.144	0.215
<i>n</i> -Nonanoate	0.106	0.116	$\text{HO}_2\text{C}\cdot[\text{CH}_2]_5\cdot\text{CO}_2\text{H}$	0.0903	0.135
<i>n</i> -Octanoate	0.068	0.075	$\text{HO}_2\text{C}\cdot[\text{CH}_2]_4\cdot\text{CO}_2\text{H}$	0.0565	0.084
<i>n</i> -Heptanoate	0.043	—	$\text{HO}_2\text{C}\cdot[\text{CH}_2]_3\cdot\text{CO}_2\text{H}$	0.0352	—
<i>n</i> -Hexanoate	0.027	—	—	—	—
<i>n</i> -Valerate	0.0178	—	—	—	—

with this technique to elucidate the structure of a variety of mono-, di- and tetra-unsaturated acids occurring in natural fats.

In Fig. 4 is shown the result obtained by oxidizing a small quantity of mono-unsaturated C_{18} acid collected from a gas chromatogram of the acids present in human red cells and plasma (James & Lovelock, 1957). The main products are clearly azelaic and *n*-nonanoic acids, showing oleic acid to

Fig. 3. Relationship between log (time of emergence) and number of carbon atoms in the molecule for mono- and dicarboxylic acids in the paraffinic stationary phase (Apiezon M). ▲, Dicarboxylic acids; ●, monocarboxylic acids.

be the main constituent. The small amount of *n*-octanoic acid present is probably derived by further oxidation of the nonanoic acid, but the fact that *n*-heptanoic acid is present in greater quantity rules out octanoic acid as its precursor. Clearly the heptanoic acid itself must represent a primary oxidation product, and in confirmation a peak of the C_{11} dicarboxylic acid (which cannot be derived from azelaic acid) can be seen. Thus vaccenic acid (octadec-11-enoic acid) is also present in the mono-unsaturated C_{18} fraction.

In Fig. 5 are shown the acids resulting from the oxidation of a diunsaturated acid present in human plasma phospholipids and isolated with the gas chromatogram. The main components are azelaic acid and hexanoic acid, so that the original acid must be octadeca-9:12-dienoic acid (linoleic acid). The presence of a small amount of *n*-nonanoic acid indicates contamination of the sample with oleic acid.

Fig. 5. Result of oxidation of front half of peak diunsaturated C_{18} acid from a chromatogram of the acids of human-plasma phospholipids. Conditions as for Fig. 1. Peak identification: (1) air, (2) methyl *n*-hexanoate, (3) methyl *n*-nonanoate, (4) dimethyl suberate, (5) dimethyl azela-ate.

Fig. 4. Result of oxidation of the rear half of the mono-unsaturated C_{18} peak from a chromatogram of acids from human-plasma phospholipids. Conditions as for Fig. 1. Peak identification: (1) air, (2) methyl *n*-hexanoate, (3) methyl *n*-heptanoate, (4) methyl *n*-octanoate, (5) methyl *n*-nonanoate, (6) dimethyl pimelate, (7) methyl *n*-decanoate, (8) dimethyl suberate, (9) dimethyl azela-ate, (10) dimethyl sebacate, (11) dimethyl hendecanedioate, (12) methyl *n*-tetradecanoate.

Fig. 6 demonstrates the complex of acids obtained by oxidation of a composite peak of mono-unsaturated C_{18} acids isolated from cow's milk. The following monocarboxylic and dicarboxylic acids are clearly primary oxidation products: *n*-pentanoic, *n*-heptanoic, *n*-nonanoic, *n*-tetradecanoic and succinic, glutaric, azelaic, hendecanedioic and tridecanedioic acids. The corresponding mono-unsaturated acids are therefore octadec-13-, -11-, -9- and -4-enoic acids. The occurrence of glutaric acid would also suggest some octadec-5-enoic acid to be present, but as only a trace of *n*-tridecanoic acid can be seen in the chromatogram no definite conclusions can be drawn. It should be noted that small amounts of all the mono- and di-carboxylic acids are produced by oxidation of the very small amounts of stationary phase emerging from the column that contaminate all the collected fractions.

In Table 2 is given a list of some mono- and di-unsaturated acids (isolated from natural sources) whose structures have been determined by this technique.

*Unsaturated acids containing more
than two double bonds*

In a study of the fatty acids present in human red cells and plasma phospholipids (James & Lovelock, 1957) a series of unsaturated acids moving more slowly than stearic acid were discovered. The acids had retention volumes relative to methyl stearate of 1.25, 1.47, 1.64 and 1.88. Methyl nonadecanoate would be expected to have a relative retention volume of 1.54, and for this reason the first two acids were tentatively assumed to be unsaturated C_{19} acids. The slower of these two acids (relative retention volume 1.47) was, however, present in amounts similar to those reported for arachidonic acid (eicosa-5:8:11:14-tetraenoic acid).

In order to establish the chain length of this acid, 200 mg. of acids obtained from hydrolysis of the phospholipids of human red cells was separated on a reversed-phase liquid-liquid chromatogram (Howard & Martin, 1950). The fractions corresponding to the C_{12} , C_{14} , C_{16} and C_{18} saturated acids were methylated and run on the gas-liquid chromatogram. The C_{14} fraction contained myristic acid, linoleic acid and the unsaturated ' C_{19} ' acid, all of which were easily separated in milligram amounts on the gas column. The ' C_{19} ' peak was collected and reduced with hydrogen over palladium on barium sulphate. The reduced acid when re-run on the gas chromatogram gave two peaks, the slower of which was in the position expected for *n*-eicosanoic acid (retention volume relative to methyl stearate, 2.34). The other acid moving in front of this was presumably a mono-unsaturated acid produced by incomplete reduction. On this basis the ' C_{19} ' acid can be assigned a chain length of 20 carbon atoms.

Fig. 6. Result of oxidation of mono-unsaturated C_{18} acids from cow's milk. Conditions as for Fig. 1. Peak identification: (1) air, (2) methyl *n*-pentanoate, (3) methyl *n*-hexanoate, (4) dimethyl succinate, (5) methyl *n*-heptanoate, (6) dimethyl glutarate, (7) methyl *n*-octanoate, (8) dimethyl adipate, (9) methyl *n*-nonanoate, (10) dimethyl pimelate, (11) dimethyl suberate, (12) dimethyl azela-ate, (13) dimethyl sebacate, (14) dimethyl hendecanedioate, (15) methyl *n*-tetradecanoate, (16) methyl *n*-pentadecanoate, (17) dimethyl tridecanedioate.

Table 2. *Identification of the structure of some naturally occurring mono- and di-unsaturated acids*

Source	Nature of acid	Fragments identified	Structure
Cod-liver oil	C ₁₆ mono-unsaturated	<i>n</i> -Heptanoic, azelaic	Hexadec-9-enoic
Human sebum	C ₁₄ mono-unsaturated	Glutaric	Tetradec-5-enoic
Human sebum	C ₁₆ mono-unsaturated	<i>n</i> -Decanoic	Hexadec-6-enoic
Cardiolipin	C ₁₆ mono-unsaturated	<i>n</i> -Heptanoic, azelaic	Hexadec-9-enoic
Cardiolipin	C ₁₈ di-unsaturated	<i>n</i> -Hexanoic, azelaic	Octadeca-9:12-dienoic
Faecal fatty acids	C ₁₈ mono-unsaturated	<i>n</i> -Nonanoic, azelaic	Octadec-9-enoic

This unsaturated acid when subjected to oxidative degradation gave three acids: *n*-hexanoic, malonic and glutaric acids. It was thus identified as arachidonic acid (Mowry, Brode & Brown, 1942).

SUMMARY

1. The separation of the methyl esters of mono- and di-carboxylic acids on the gas-liquid chromatogram is described.

2. Oxidation with permanganate in acetic acid followed by identification of the split products on the gas-liquid chromatogram is used to establish the structures of some naturally occurring mono- and di-unsaturated acids.

3. The presence of vaccenic acid in human plasma is confirmed by this technique and cow's milk is shown to contain a variety of mono-unsaturated octadecenoic acids.

4. The isolation of an unsaturated acid present as a major component in human red cells and plasma is described, and is shown by reduction and oxidation to be arachidonic acid.

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Pyridoxamine Phosphate Transaminase

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The starting material for the preparation of tryptophanase in these laboratories has been a 0.8M-potassium chloride extract of cells of *Escherichia coli*. Happold & Struyvenberg (1954) showed that when this was resolved into apoenzyme and co-enzyme (pyridoxal phosphate) by dialysis against water, ammonium, potassium or rubidium ions were also required for reactivation, but that when the holoenzyme was dialysed against 0.25M-potassium chloride the dissociation of apoenzyme and coenzyme occurred only very slowly, over a period of days.

In later studies on this enzyme we observed that aqueous extracts were devoid of tryptophanase activity but did contain apotryptophanase, and that this was remarkably stable; it naturally requires pyridoxal phosphate and potassium, ammonium or rubidium ions for activation. The further observa-

tion that pyridoxamine phosphate also caused an increase in tryptophanase activity was, however, an unexpected discovery; our previous failure, and that of Wood, Gunsalus & Umbreit (1947), to observe this fact may have been due to the use of ammonium sulphate in the partial purification of the enzyme or, in our experiments, to the use of potassium chloride solutions as the enzyme solvent. We now suggest that, whilst pyridoxal phosphate is the coenzyme of tryptophanase, as of so many other enzymes associated with the intermediary metabolism of amino acids, a specific transaminase exists which catalyses the reversible interconversion of pyridoxamine phosphate and pyridoxal phosphate. We have called this enzyme pyridoxamine phosphate transaminase (Beechey & Happold, 1955). The importance of this reaction in the physiology of the cell needs no emphasis.