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The Constitution of Hexadecatrienoic Acid from the Glycerides of Rape (*Brassica napus* L.) Leaf

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Apart from hexadec-9-enoic acid, the C₁₆ unsaturated acids are of somewhat rare occurrence. From sardine oil there has been isolated hexadeca-6:10:14-trienoic acid (Toyama & Tsuchiya, 1935) and a hexadecatetranoic acid (Tutiya, 1940, 1941). More recently Shorland (1945) reported the presence of hexadecatrienoic acid in the glycerides of rape leaf.

The purpose of the present work is to determine more precisely the constitution of the hexadecatrienoic acid found in rape-leaf glycerides. In addition, the method of Armstrong & Hilditch (1925), commonly used to locate the position of the double bonds by disruptive oxidation, has been examined using linolenic acid, the constitution of which has already been determined by previous workers (cf. Erdmann, Bedford & Raspe, 1909). This step has been deemed advisable in view of difficulties which have occasionally been experienced by other workers in the isolation of the oxidation products from polyethenoid acids (cf. Shinowara & Brown, 1940).

EXPERIMENTAL

Oxidation of ethyl linolenate

Ethyl linolenate used for this work was prepared as described by McCutcheon (1938, 1940). Spectroscopic analysis by the method of Hilditch, Morton & Riley (1945) showed the presence of 95.5% (0.2% conjugated) triene acids and 2.7% (1.7% conjugated) diene acids, while the iodine value 245.8 (theoretical for ethyl linolenate 248.9) also confirmed the purity of the preparation.

The ester (8.1 g.) was dissolved in 140 ml. of acetone, and

finely divided KMnO₄ (75 g.) was added (cf. Armstrong & Hilditch, 1925) gradually under reflux until vigorous reaction ceased. The mixture was then boiled for a further 4 hr.

After removal of the acetone on a water bath, the residue was extracted with 600 ml. of hot 5% (w/v) KOH and the extract concentrated to 150 ml. (cf. Haworth, 1929). The extract was acidified with dil. H₂SO₄ to congo red, and the volatile acids separated by steam distillation. By comparing the amount of 0.1 N-KOH required for neutralization with the dried weight (3.41 g.) of the K salts (cf. Dolby, Nunn & Smedley-MacLean, 1940) the equivalent of the volatile acids was found to be 71.3 as compared with 74.0 required for propionic acid. To test the validity of the method a sample of propionic acid was steam distilled as above and gave an equivalent of 74.9.

After extraction of the non-volatile residue with ether, the extract was boiled with water and filtered to remove any oily insoluble matter. Recrystallization from hot water yielded 1.53 g. crystals, equiv. 93.1, m.p. 104.5–106.5°. From the mother liquors a further crop of crystals (1.42 g., equiv. 91.0) was obtained. By applying counter-current distribution methods (Craig, 1944) to 0.117 g. of this latter fraction, using a mixture of 4:3 by volume ether-light petroleum and 20% aqueous ethanol, there was obtained, in addition to azelaic acid, 0.016 g. of suberic acid, equiv. 87.8, m.p. 131–137° (not depressed by admixture with authentic suberic acid).

Other similar oxidation experiments with ethyl linolenate as well as with methyl oleate also indicated that in addition to azelaic acid there were produced small but variable amounts of suberic acid.

Oxalic acid was estimated as described by Haworth (1929), the yield amounting to 60% of the theoretical. Malonic acid was not detected, indicating presumably, as was found by Haworth (1929), that under these conditions this acid is further oxidized to yield oxalic acid.

Oxidation of methyl hexadecatrienoate

The acids (a) 33.0 g. and (b) 35.4 g. from the glycerides of two separate samples of rape leaf were submitted to an acetone separation (10 vol. three times at -30°), and the soluble acids (a) 29.6 g. and (b) 32.1 g. converted to methyl esters. The esters were fractionated in an electrically heated, packed 60 cm. column of the type described by Longenecker (1937), and gave the results shown in Table 1.

Table 1. *Fractionation of methyl esters of acids from rape leaf*

(a)				
Fraction no.	Wt. (g.)	B.p./about 0.1 mm. ($^{\circ}$)	Saponification equivalent	Iodine value
1	1.79	101-105	242.6	105.3
2	1.78	105-107	264.1	206.4
3	2.32	107-114	269.0	200.3
4	2.28	114-125	275.3	186.8
5	2.51	125-127	289.8	217.4
6	2.46	127	289.9	223.8
7	2.74	127-130	292.1	227.3
8	3.25	130	292.6	233.3
9	3.53	130	292.9	229.3
Residue	4.56	—	346.5	166.6
Total weight	27.82	—	—	—

(b)				
Fraction no.	Wt. (g.)	B.p./about 0.2 mm. ($^{\circ}$)	Saponification equivalent	Iodine value
1	1.35	114-118	245.2	119.5
2	1.90	118-123	264.2	187.4
3	1.62	123-125	269.8	189.5
4	1.89	125-135	278.7	194.5
5	2.30	135-139	283.6	206.2
6	3.45	139	285.5	210.3
7	2.43	139	287.4	213.0
8	3.27	139-141	289.5	213.4
9	3.21	141-142	290.2	218.5
10	1.80	142	289.0	217.2
Residue	5.86	—	370.0	157.4
Total weight	29.08	—	—	—

Fractions 2-4 from (a) and 2-5 from (b) were combined separately and crystallized from 15 vol. of methanol at -15° ; the soluble portions on fractionation gave the results shown in Table 2. Fractions 2-4 from (a) and 2 and 3 from (b) (Table 2) were further separated by crystallization of the corresponding acids from 10 vol. of acetone at -78° . The bulked acetone-soluble acids (4.2 g.) had a saponification equivalent of 250.7 and an iodine value of 277.1. (Theoretical values for hexadecatrienoic acid, saponification equivalent 250.0, iodine value 304.6.) Hydrogenation of 0.18 g. of the methyl esters, as described by Reasenberg, Lieber & Smith (1939), yielded on crystallization from methanol at -15° , 0.17 g. insoluble esters, m.p. $28.5-29.5^{\circ}$, not depressed by admixture with genuine

Table 2. *Fractionation of acetone-soluble methyl esters*

(a)				
Fraction no.	Wt. (g.)	B.p./about 0.1 mm. ($^{\circ}$)	Saponification equivalent	Iodine value
1	0.16	76-102	—	216.4
2	0.56	102-107	261.1	219.0
3	0.54	107	266.2	244.4
4	1.20	107-121	264.7	243.3
Residue	1.21	—	283.9	189.1
Total weight	3.67	—	—	—

(b)				
Fraction no.	Wt. (g.)	B.p./about 0.1 mm. ($^{\circ}$)	Saponification equivalent	Iodine value
1	0.26	76-98	255.5	108.4
2	1.69	98-106	262.9	225.5
3	1.37	106-115	266.5	218.8
4	0.78	115-120	281.7	207.4
Residue	2.29	—	293.4	208.5
Total weight	6.39	—	—	—

methyl palmitate. Spectroscopic measurements before and after alkali isomerization (Mitchell, Kraybill & Zscheile, 1943), after allowing for the differences in molecular weight between C_{18} and the C_{18} unsaturated acids, indicated the presence of

Fig. 1. Absorption spectrum of methyl hexadecatrienoate before (----), and after (—) isomerization, according to the method of Mitchell, Kraybill & Zscheile (1943).

85.9% (0.2% conjugated) triene acids and 3.3% (1.8% conjugated) diene acids. The absorption curve is shown in Fig. 1.

Two separate batches (c) 1.86 g. and (d) 2.03 g. of methyl esters from the C_{16} highly unsaturated acids were oxidized and the equivalents (c) 70.9 and (d) 71.3 of the volatile acids determined as for ethyl linolenate. From the anhydrous acids (1.05 g., equivalent 71.0) prepared by distillation of the dried potassium salts from phosphoric acid saturated with P_2O_5 there was obtained using the method of Kuehn & McElvain (1931), 0.74 g. of anilide. Recrystallization of the anilide from water yielded 0.55 g. propionanilide, m.p. 104–104.5°, not depressed by admixture with authentic propionanilide.

Extraction of the non-volatile acids with ether yielded (c) 0.82 g. and (d) 0.99 g. of impure acids.

Attempts were made, without marked success, to purify (c) by counter-current distribution and by chromatographic adsorption from acetone solution using a column of anhydrous sodium carbonate. The latter technique, however, resulted in the separation of some impure acid, m.p. 96–98°, raised to 99.5–102° on admixture with genuine pimelic acid (m.p. 104.5–105.5°). Crystallization of 0.55 g. (c) from benzene followed by sublimation *in vacuo* and recrystallization from the same solvent yielded 0.42 g. acid, equivalent 79.3, m.p. 91–96.5°.

By similar procedure, but recrystallizing twice from benzene, 0.99 g. (d) yielded 0.62 g. acid, equivalent 79.1, m.p. 95–98°, which was raised to 100.5–103.5° when mixed with pimelic acid. The results thus indicate the presence of pimelic acid, but further attempts at more complete purification by crystallization were unsuccessful.

The amounts of oxalic acid determined, as previously described, represented (c) 72% and (d) 68%, respectively, of the yields that would be expected from a compound with three double bonds separated by two methylene groups.

DISCUSSION

The extensive work of Green & Hilditch (1937) on the oxidation of hydroxy acids with aqueous alkaline permanganate indicates that although this technique yields mainly azelaic acid in the case of the $\Delta^{9,10}$ -polyethenoid acids, nevertheless, there are produced in addition by no means negligible amounts of suberic acid. For this reason they suggest that oxidation with aqueous alkaline permanganate compares unfavourably with oxidation by ozone or by potassium permanganate in acetone solution as a method for the location of the double bonds. The present results generally confirm the constitution of linolenic acid as first proposed by Erdmann *et al.* (1909) in that the expected products of oxidation, namely, propionic, oxalic and azelaic acids have been obtained. Nevertheless, the indication in the present work of substantial amounts of suberic acid in the

oxidation products from ethyl linolenate as well as in the oxidation products from methyl oleate suggests that the objection raised by Green & Hilditch (1937) to the use of aqueous alkaline potassium permanganate may also apply, though to a lesser extent, when the oxidation is carried out with potassium permanganate in hot acetone solution. While admitting the possibility that the Haworth (1929) alkali extraction procedure used in this work for ethyl linolenate might have promoted the formation of suberic acid, the presence of this acid in the oxidation products of methyl oleate could not have arisen in this way, since the extraction of the oxidation products was carried out exactly as described by Armstrong & Hilditch (1925). It is therefore apparent that even when the latter method is used there is need for some caution in its use for the determination of the structure of unknown acids.

The present work confirms the earlier observations of Shorland (1945) in regard to the occurrence of a hexadecatrienoic acid in the rape-leaf glycerides. Allowing for the general nature of the oxidation of polyethylenic acids with potassium permanganate in acetone solution, the yields of propionic, pimelic and oxalic acid obtained from this hexadecatrienoic acid, together with the spectroscopic evidence before and after alkali isomerization, establish the constitution of the acid as hexadeca-7:10:13-trienoic acid, and distinct from hiragonic acid, hexadeca-6:10:14-trienoic acid, isolated by Toyama & Tsuchiya (1935) from sardine oil.

Hilditch (1948) has discussed the structural relationships of the natural unsaturated higher fatty acids and has indicated the fact that most natural straight-chain acids share a somewhat singular partial resemblance to oleic acid in that one or other part of their molecules is identical with that of one-half of the oleic acid molecule. The C_{16} unsaturated acid from rape-leaf glycerides would therefore appear to have a somewhat unusual structure, but one which nevertheless resembles linolenic acid, of which it could be precursor or from which it could be derived by β -oxidation.

In general, it has been found that the few leaf fats which have so far been examined are more or less similar in composition containing as the main constituent, linolenic acid (cf. Shorland, 1944, 1945). Although C_{16} highly unsaturated acids have been searched for in other leaf fats so far examined by one of us (Shorland, 1950) these acids have not been found, nor have they been detected in the phosphatide fraction of the rape.

SUMMARY

1. Oxidation of ethyl linolenate by potassium permanganate in acetone solution generally confirmed the structure proposed by other investigators,

but in addition to azelaic acid there was also produced some suberic acid.

2. Oxidation with potassium permanganate in acetone solution of methyl hexadecatrienoate isolated from rape-leaf glycerides yielded pimelic,

oxalic and propionic acids. This evidence in association with the spectroscopic results before and after isomerization show that this acid has the formula

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Further Observations on the Action of Chymotrypsin on Insulin

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The action of crystalline chymotrypsin on insulin has recently been described (Butler, Dodds, Phillips & Stephen, 1948) and the biological activity of the products has been investigated (Phillips, 1951). The degradation of the hormone by this enzyme gave a large fragment, the core, of molecular weight about 5000 (Butler, Phillips, Stephen & Creeth, 1950) and a mixture of small peptides which have been partially separated by paper chromatography (Phillips, 1949).

In this paper the degradation has been further studied: (a) to see whether the products of proteolysis cause any inhibition of the enzyme, (b) to locate the pH optimum for the reaction (this has been found to have two optima) and (c) to define the general character of the reaction, which has been found to be of the 'all-or-none' type of Tiselius & Eriksson-Quensel (1939).

EXPERIMENTAL

Insulin. Crystalline bovine insulin from Boots Pure Drug Co. was used, assaying 22–23 units/mg.

Chymotrypsin. Part of a sample of α -chymotrypsin kindly given by Dr M. Kunitz was used. The digestions were carried out in a similar way to the previous work, using approximately 3×10^{-4} t.u. (trypsin units) of enzyme/5 mg. insulin

in 1 ml. of buffer at 25°. The trypsin unit referred to here is the quantity of enzyme which will liberate 1 mmol. of tyrosine from haemoglobin in 1 min. at 35.5°. According to Northrop, Kunitz & Herriott (1948) 0.038 trypsin unit is equivalent to 1 mg. of protein N.

Nitrogen. Amino N, non-protein N (N.P.N.) and total N were determined as in the previous papers. N.P.N. is expressed as % of total N.

Enzyme activity. This was determined by the method of Anson (1938).

Tyrosine. The colour given with the phenol reagent of Folin & Ciocalteu (1927) was measured, there being no tryptophan in insulin to interfere with it.

Insulin activity. This was determined by measuring the blood sugar levels (Folin & Wu, 1929) immediately prior to and 1, 2 and 3 hr. after subcutaneous injection into 1.5–2.5 kg. rabbits which had been starved 18 hr., and which had recently been shown to give a 40–60% maximum fall when given 1 unit of insulin.

RESULTS

The effect of the products of digestion of insulin by chymotrypsin on the activity of the enzyme

This effect has been estimated in three ways: by the remaining enzyme activity in the digest when tested against a standard substrate (haemoglobin); by digestion of insulin in the presence of urea; and