Structures of the ester-linked mono- and diunsaturated fatty acids of pig brain

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SUMMARY The structural characterization of mono- and diunsaturated fatty acids present in ester form in pig brain is described. The acids were isolated by mild saponification of total lipids and esterified. The esters were then separated using column, thin-layer, and gas-liquid chromatography, and the positions of the double bonds were determined by oxidative ozonolysis. Forty-seven acids were found, 25 of which have not been previously reported to occur in natural sources. These include the even- and odd-numbered monoenoic acids and the even-numbered dienoic acids 16 to 22 carbon atoms long. Each acid proved to consist of three to six positional isomers. Judging by the positions of the double bonds, the longer monoenoic acids appear to be formed by chain elongation with C_2 units from shorter unsaturated acids: $16:1^9$ (palmitoleate), $16:1^6$, $17:1^9$, $18:1^9$ (oleate), and $19:1^9$. The dienoic acids appear to be formed from linoleate, palmitoleate, oleate, and $16:1^6$.

RECENT STUDIES have shown that the cerebroside saturated acids are formed by two processes: chain lengthening of shorter acids and 1-carbon degradation of longer acids (1–5). The non-hydroxy saturated acids are also converted to 2-hydroxy acids (6, 7). That the chain-lengthening enzymes in brain act also on unsaturated acids was shown with labeled oleate (8). By examining the structures of the positional isomers of the non-hydroxy unsaturated sphingolipid acids, we have shown the existence of families of acids that appear to be formed by the same enzyme systems that operate on the saturated acids (9). A similar series of families was found in the hydroxy unsaturated acids, suggesting these are formed from the non-hydroxy unsaturated acids (10). The structures of the non-hydroxy dienoic sphingolipid acids indicated that the brain could insert a second double bond into a monoenoic acid, as well as utilize the essential fatty acid, linoleate.

The major families found (identified by counting the number of carbon atoms from the ω-end of the chain to the double bond) were the ω7 and ω9 families, probably arising by lengthening of palmitoleate and oleate, respectively. Dienes in the ω6 family are evidently formed from linoleate. However, members of the ω8 and ω10 families were also found and the question arose as to whether shorter members of these families exist. An additional question is whether the same families of acids exist in ester lipids as well as in sphingolipids. Accordingly, we have made a detailed study of the structures of the mono- and dienoic fatty acids bound in ester form in brain.

EXPERIMENTAL METHODS

Most of the methods and materials have been described previously (9, 10). The total lipid of two pig brains (total weight 196 g) was saponified with 1 N ethanolic KOH for 60 min at 38° (11). After removal of non-saponifiable lipids with hexane, the fatty acids were taken up in ether and esterified. The methyl esters were then purified with a 100-g silica gel column, elution being made with 2,000 ml of hexane–benzene 7:3. The mono- and diunsaturated esters were obtained in two groups with the aid of silver nitrate-impregnated silica gel and mercuric acetate adduction. The yields of monoenes and dienes were 2.03 g and 101.4 mg respectively.

The individual members within each group were isolated by GLC using a preparative SE-30[1, 2] column.

[1] Abbreviations used: DEGS is a polymeric form of diethyleneglycol succinate, SE-30 is a General Electric silicone gum, GLC is gas-liquid chromatography, and TLC is thin-layer chromatography.

In fatty acid abbreviations, the first number indicates the chain length, the second number indicates the number of double bonds, the superscripts indicate the positions of the double bonds, h indicates a 2-hydroxyl group. The Greek letter ω is used to signify the position of a fatty acid double bond, counting from the ω-end.

[2] Obtained from Wilkens Instrument and Research, Inc., Walnut Creek, California.

The methyl esters ranging between 16 and 22 carbon atoms were collected and passed through a silica gel column to remove material that bled from the SE-30. As TLC[1] showed the monoenes were still impure, they were saponified to remove non-saponifiable lipid, esterified, and repurified on a Florisil[3] column by elution with hexane. The odd-numbered esters were purified further with GLC, as they were contaminated appreciably by the much more plentiful even-numbered esters.

Each ester was then characterized by infrared spectrophotometry and analyzed for positional isomers by ozonolysis and GLC of the fragments.

RESULTS

Purity of the Isolated Methyl Esters. All the purified esters, which were colorless oils, gave single spots on TLC except 22:1, in which case a faint, faster spot was observed. Analysis by GLC with a DEGS column showed no impurities except in the 16:2 and the odd-numbered acids, in which case some homologues were detected, but totalled less than 1% of the major component.

The infrared spectra were normal for *cis* monoenes and dienes (9), but a small peak at 10.17 μ was observed with 21:1. This peak might be due to some *trans* unsaturated material, although the *trans* peak is usually nearer 10.3 μ.

The major impurity seen in TLC of the crude 17:1 fraction was just below the methyl ester spot before saponification and just above afterward. The infrared spectrum of the non-saponifiable material thus obtained suggested the presence of a *cis*-unsaturated ketone. In contrast, the major impurity in the 19:1 fraction was not affected by saponification. Its spectrum strongly resembled that of an alkenyl ether (12).

While TLC disclosed impurities in the initial diene fraction, these were removed during the GLC isolation.

Occurrence of Positional Isomers. Table 1 lists the monounsaturated fatty acid esters isolated and their positional isomers. Each component proved to consist of three to six isomers, the abundances of which are shown in the 4th and 5th columns. As noted in the sphingolipid acids, members of the ω7, ω8, ω9, and ω10 families were found. In addition, there are members of the ω11, ω12, and ω13 families. Not shown is the presence of a trace amount of the ω6 family in the odd-numbered acids.

As might be expected, oleic acid (18:1[9]) is the most common acid in brain. The large amounts of *cis*-vaccenic acid (18:1[11]) and of the 20:1 acids were somewhat unexpected.

[3] Floridin Co., Tallahassee, Florida.

TABLE 1 MONOENOIC FATTY ACIDS OF PIG BRAIN ESTERS AND THEIR RELATIVE AND ABSOLUTE ABUNDANCES

Ester	Position of Double Bond: From COOH	Position of Double Bond: From CH_3	Proportion of Each Isomer	Concentration in Fresh Brain	Characterized Previously
			%	*mg/100g*	
16:1	6	10	31	9.1	Human hair (13)
	7	9	22	6.5	Bacteria (27)
	9	7	47	14	Brain* (13)
17:1	6	11	12	0.3	Human hair (13)
	7	10	3	0.08	
	8	9	17	0.5	Human hair (13)
	9	8	63	1.8	Lamb caul (13)
	10	7	4	0.1	
18:1	8	10	3	18	Human hair (13)
	9	9	80	480	Brain* (13)
	11	7	17	100	Brain* (13)
19:1	8	11	7	0.1	
	9	10	21	0.3	
	10	9	7	0.1	Shark liver (28)
	11	8	59	0.8	Shark liver (28)
	12	7	5	0.07	
20:1	10	10	2	2.4	
	11	9	68	82	Brain* (13, 29, 30)
	13	7	30	36	
21:1	9	12	8	0.06	
	10	11	7	0.05	
	11	10	13	0.1	
	12	9	7	0.05	
	13	8	55	0.4	
	14	7	7	0.05	
22:1	9	13	16	2.0	
	10	12	3	0.4	
	12	10	3	0.4	
	13	9	49	6.2	Spinal cord (13, 30)
	15	7	29	3.7	

* As well as many other sources.

Table 2 shows similar data for the dienes. Here too we see the same families that were found in the sphingolipid acids. Particularly striking is the occurrence of 16:2 isomers.

Small amounts of 24:1, 26:1, 17:2, and 19:2 were observed during GLC but they were not collected.

Of the 47 acids listed, 22 have been characterized before and their site of occurrence is shown in the 6th column of each table. To simplify the problem of bibliographic reference, the chapter by Markley (13) is cited wherever possible.

DISCUSSION

Positional Isomers of the Monoenoic Acids. The ω7 family is seen in the fatty acids of all chain lengths, both even- and odd-numbered. As suggested in the case of the sphingolipid acids, the even-numbered acids may be synthesized by addition of acetate residues onto the

TABLE 2 DIENOIC FATTY ACIDS OF PIG BRAIN ESTERS AND THEIR RELATIVE AND ABSOLUTE ABUNDANCES

Ester	Position of Double Bond: From COOH	Position of Double Bond: From CH_3	Proportion of Each Isomer	Concentration in Fresh Brain	Previously Characterized
			%	*mg/100g*	
16:2	3, 6	10, 13	2	0.01	
	4, 7	9, 12	80	0.32	
	5, 8	8, 11	5	0.02	
	6, 9	7, 10	8	0.03	Herring oil (19, 31)
	7, 10	6, 9	6	0.02	Herring oil (19)
18:2	5, 8	10, 13	3	0.5	
	6, 9	9, 12	13	2.2	Liver (19, 32)
	8, 11	7, 10	8	1.3	Rat tissues (33)
	9, 12	6, 9	76	13	Brain* (13)
20:2	7, 10	10, 13	4	0.5	
	8, 11	9, 12	72	9.1	Ox, rat tissues (32, 34)
	10, 13	7, 10	6	0.8	
	11, 14	6, 9	18	2.3	Brain* (29)
22:2	9, 12	10, 13	20	0.5	
	10, 13	9, 12	63	1.5	Beef liver (35)
	12, 15	7, 10	9	0.2	
	13, 16	6, 9	8	0.2	Seed oil (13)

* As well as many other sources.

primary acid, $16:1^{9}$, and the odd-numbered members may be formed by 1-carbon degradation of the longer homologous acids. If this is so, it would appear that the 1-carbon degradation enzymes act to produce acids used for ester lipid synthesis as well as sphingolipid synthesis. However, the generality of distribution of these enzymes is still unclear, for there is so little $17:1^{10}$, $19:1^{12}$, and $21:1^{14}$ in brain that these acids might well be formed only in the cells that make sphingolipids (14).

Similar reasoning applies to the members of the ω9 family, presumably formed from oleate. In this case, the existence of $16:1^{7}$ is unexpected; it might be formed from oleate by β-oxidation (15) or by desaturation of 16:0.

Members of the ω10 family appear in all the monoenes, the proportion being minor except in 16:1 and 19:1. Perhaps $19:1^{9}$ is the precursor of $21:1^{11}$, and $16:1^{6}$ is the precursor of all the other family members. The $19:1^{9}$ might be formed by desaturation of 19:0 by the same enzyme that converts stearate to oleate, and the 16:1 might be formed by desaturation of 16:0. In terms of actual brain content, $16:1^{6}$ is much more plentiful than $19:1^{9}$.

Unlike the above three families, the members of the ω8 family are found only in the odd-numbered acids, where they are the major isomers. It seems likely that the precursor of this series is the shortest one found, $17:1^{9}$. This acid is probably formed by the desaturating enzyme that forms oleate and palmitoleate. The absence of the ω8 series in the even-numbered acids does not necessarily mean that the 1-carbon degradation enzymes do not act on these isomers. The actual amount per brain of these members is so small that the amount of even-numbered members that might be formed would be too small to detect. Mead and Levis have recently shown that the odd-numbered acid, 23h:0, can be degraded to 22:0 (3).

In the sphingolipid non-hydroxy and hydroxy monoenes, the ω8 family was found to be less dominant. This probably reflects a greater rate of synthesis of these acids by 1-carbon degradation.

The ω11 series, like the ω8, also occurs just in the odd-numbered acids. If $17:1^{6}$ is the precursor of this series, it may be formed by the same enzyme that converts 16:0 to $16:1^{6}$. This family was not seen in the sphingolipid acids, from which one might conclude that the desaturating enzyme does not occur in the cells that make sphingolipids.

The ω12 series is found only in 22:1 and 21:1 and the ω13 series only in 22:1. Neither series appeared in the sphingolipid acids. The composition of 22:1 in ester-linked and sphingosine-linked fatty acids is very different, the latter containing far more of the ω7 family. This is consistent with the idea that the two types of lipids are made largely in different brain cells (14).

Although not included in Table 1, traces of isomers in the ω6 family were seen in the odd-numbered acids. These could arise from $15:1^{9}$, which has been reported to occur in beef muscle and tallow (16).

The Dienoic Acid Isomers. The most common dienoic acid in nature appears to be linoleate ($18:2^{9,12}$). In agreement with other laboratories (11, 17, 18), we found the concentration in brain to be much less than in other tissues. Presumably the brain gets this acid from the diet, for animals seem unable to synthesize it from acetate. It may well be that the blood-brain barrier acts to reduce the uptake of linoleate from the blood.

Isomers in the ω6,ω9 family were found in all the dienes, suggesting that linoleate is lengthened by acetate residues. The occurrence of 16:2 in this family might be due to β-oxidation of linoleate, which is known to undergo oxidation in the whole rat (15). However, $16:2^{7,10}$ occurs also in herring oil (19) and thus might enter exogenously.

The ω7,ω10 family occurs in all the dienes as well as in the sphingolipid dienes. It is likely that this is derived from palmitoleate by chain lengthening and desaturation. This is the major isomer in 26:2 (9).

Similarly, the ω9,ω12 family is found in all the dienes, presumably formed from oleate. This is the major isomer in the ester-linked 16:2, 20:2, and 22:2. While several members of this family probably occur in diets, the large

difference in distribution between the linoleate family and the ω9,ω12 family suggests the latter is endogenous. However, the ω9,ω12 isomer constitutes a very large proportion of the 16:2 group, so that this acid might be the precursor of all the others in this family, in which case it might be derived from an unknown dietary source.

The ω10,ω13 family is represented at all chain lengths but is a very small component in all except 22:2. Perhaps it is formed from $16:1^6$ by lengthening and desaturating since this acid is by far the most common acid in the ω10 family.

That polyenoic acids can be synthesized from palmitoleate and oleate has been shown with in vivo and in vitro (liver) experiments (20, 21). The synthesis seems to be stimulated by a diet deficient in essential fatty acids (22), and one might conclude from the relative abundances of these families in the brain dienes that the brain is normally in a state of deficiency. The low level of linoleate in brain suggests this is true. On the other hand, brain is rich in the essential tetraene, arachidonate, possibly because the more highly unsaturated acid can more easily penetrate the blood-brain barrier. This suggestion is supported by the finding that considerable penetration of pentaenoic acids into brain mitochondria takes place in rats on a cod liver oil diet (18).

Desaturation vs Chain Lengthening. Every isomeric monoene found in the brain could conceivably arise by enzymatic dehydrogenation of the corresponding saturated acid. There could be a single desaturating enzyme that attacks fatty acids anywhere between the 7th and 13th carbons, counting from the *methyl* end, but then one would have to postulate a variety of other enzyme reactions that are more specific in order to explain the variations observed in the actual proportions of the different isomers and families. We suggest instead that there are two desaturating enzymes, one acting on the position 9 from the *carboxyl* end and one acting 6 from the carboxyl end. The former enzyme could then account for palmitoleate, oleate, $17:1^9$, and $19:1^9$. These four acids, by chain lengthening, 1-carbon oxidation, and 2-carbon (β) oxidation, could account for all the members of the ω7, ω8, ω9, ω10, ω12, and ω13 families except $16:1^6$. The enzyme acting on C-6 (from the *carboxyl* end) would explain the occurrence of the ω10 and ω11 families, starting with $16:1^6$ and $17:1^6$.

Unfortunately, little is yet known of the desaturating enzymes or their specificity with respect to chain length and double bond position. A study with rat liver has indicated there is a desaturating enzyme which acts on 14:0 and 17:0 to produce only members of the ω9 family, but this is an old report, not yet confirmed (23). An enzyme system in yeast has been shown to act on the 9 position (carboxyl end) to form oleate and palmitoleate (24).

The existence of chain lengthening among unsaturated acids has been demonstrated with labeled compounds in rats in the case of the sphingolipid monoenes (8) and the polyunsaturated acids (20), and in vitro with oleyl CoA (21). The existence of chain shortening by two carbon units is well established by in vitro work; and work with mice and rats (25, 26) has indicated stearate is not only degraded but acts also as a source of palmitate for lipid synthesis.

The importance of dietary fatty acids cannot be evaluated at present but must be considered because of the small amounts found in the case of some isomers. The actual diet of the pigs used as the source in this study is unknown and could have included an appreciable amount of bacterial or fungal products, in which case one might find some unusual acids. It remains to be seen whether the brains of other species reveal the wide variety observed here.

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