Unusual isomeric polyunsaturated fatty acids in liver phospholipids of rats fed hydrogenated oil

(essential fatty acids/*trans* acids/positional isomers/polyunsaturation)

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ABSTRACT Linoleic acid (18:2 ω 6) and linolenic acid (18:3 ω 3) are precursors of two series of essential fatty acids (EFA) formed by alternate desaturations and elongations. In EFA deficiency (EFAD), oleic acid (18:1 ω 9) and palmitoleic acid (16:1 ω 7) undergo the same reactions to form polyunsaturated fatty acids (PUFA) of other structures. Partially hydrogenated soybean oil (PHSO) contains isomeric 18:1 acids that can be converted to unusual isomers of 18:2 by liver microsomes. To test whether 18:2, 20:3, and 20:4 of unusual structure occur in phospholipids as a consequence of EFAD or ingestion of PHSO, rats were fed corn oil, an EFA-deficient diet, or PHSO to provide isomeric 18:1 acids. At 2.5 months the phospholipids were isolated from livers and converted to methyl esters, and the 18:2, 20:2, 20:3, and 20:4 fractions were isolated. The 18:2 and 20:2 fractions were ozonized, and, by using a computer solution of simultaneous equations, the structures and proportions of each isomer were calculated. The 20:3 and 20:4 fractions were analyzed by ozonolysis and capillary gas chromatography. When corn oil was fed, the major isomer in each group was 9,12-18:2, 11,14-20:2, 8,11,14-20:3, and 5,8,11,14-20:4. Patterns in EFAD- and PHSO-fed groups were more diverse, with large proportions of unusual isomers. Feeding EFA-deficient diet and PHSO induced measurable amounts of unusual PUFA at each step of the cascade, and these PUFA may compete in metabolism of normal PUFA and are substrates for oxidative formation of autacoids of unknown structure and function.

Study of metabolism of isomeric 18:1 fatty acids (FA) present in partially hydrogenated soybean oil (PHSO) to isomeric 18:2 FA and to longer-chain isomeric polyunsaturated fatty acids (PUFA) has been impeded by absence of analytical methods to measure individual isomers in mixtures. Recently, an ozonolysis-computer matrix procedure to analyze mixtures of positional isomers of 18:2 was developed (1). The method was used to identify and measure isomers of 18:2 and 20:2 in rat liver phospholipids (PL) as the consequence of essential fatty acid deficiency (EFAD) or of dietary intake of PHSO. PHSO has a range of 18:1 isomers that are substrates for desaturation to uncommon 18:2 positional and geometric isomers *in vitro* by rat liver microsomes and that may be elongated to 20:2 isomers or desaturated and elongated to uncommon isomers of 20:3 and 20:4 (2). PHSO also contains isomeric 18:2 acids formed by hydrogenation of 18:3 or by isomerization of 18:2 in the starting oil. The isomeric acids may be both positional and geometric isomers. We investigated the isomeric complexity of these PUFA, as influenced by type of dietary fat, to test whether the products of reactions that occur in microsomes with single substrates (3, 4) are detectable *in vivo*.[†] The analysis of isomers by ozo-

nolysis identifies only the positions of double bonds, not their geometric configuration.

METHODS AND MATERIALS

Diets and Isolation of PL. Male Sprague-Dawley rats were fed isocaloric semisynthetic diets containing 10% corn oil (normal), 10% hydrogenated coconut oil to induce EFAD (5), or 10% PHSO to provide isomeric 18:1 FA. EFAD diet was used to induce a high level of incorporation of endogenous PUFA into PL, and PHSO diet was fed to provide 18:1 and 18:2 isomeric substrates that may be desaturated, elongated, and incorporated into PL. By 2.5 months, only the EFAD rats developed an EFAD dermatitis with a dermal score of 2 on a scale of 0–5 (6), and with characteristic patterns of EFAD in tissue lipids. Thus, the PHSO contained sufficient linoleic acid (18:2 ω 6, ω 6 indicating unsaturation six carbons from the methyl end of the FA) to prevent dermatitis of EFAD. The rats were anesthetized with ether and killed, and livers were rapidly removed and stored at -70°C . Lipids were extracted from 2 g of liver (7); PL were isolated by TLC and converted to methyl esters, and the methyl dienoates, trienoates, and tetraenoates were separated by AgNO₃ TLC (8, 9). The C₁₈ dienes and C₂₀ dienes were isolated by HPLC on an Altex C₁₈ column using 95% methanol as mobile phase.

Ozonolysis. Ozonolysis-reduction was used to locate and measure the proportions of isomeric PUFA. Ozonolysis of linoleic acid, for example, yields a C₉ aldehyde ester (AE9) identifying the 9 double bond, a C₃ dialdehyde (AA3), and a C₆ aldehyde (A6) identifying the 12 double bond. Ten microliters of heptane solution of dienes was purged by 25–50 ml/min of 8% ozone in oxygen for 4 min in a frozen CO₂/acetone bath. After 1 μl of triphenylphosphine (100 $\mu\text{g}/\mu\text{l}$) in diethyl ether was added to reduce the ozonides to aldehydes, 2–5 μl of solution was injected onto a 50-m SP-2330 capillary gas chromatography (CGC) column (Supelco), programmed from 40 $^{\circ}\text{C}$ to 220 $^{\circ}\text{C}$ at 3 $^{\circ}\text{C}/\text{min}$. Integrated areas under aldehyde ester, dialdehyde, and aldehyde peaks, and their elution times, were listed in a spread sheet that corrected for ionizable carbon atoms and expressed composition as mol % within aldehyde ester, dialdehyde, and aldehyde classes.

Measurement of Conjugatable Dienes. Conjugatable dienes in 18:2 and 20:2 fractions were measured by a modified alkaline conjugation-gas chromatography procedure (10). Ra-

Abbreviations: PHSO, partially hydrogenated soybean oil; FA, fatty acids; EFA, essential fatty acids; EFAD, essential fatty acid deficiency; PUFA, polyunsaturated fatty acids; PL, phospholipids; CGC, capillary gas chromatography; AE n , aldehyde ester with n carbon atoms in the aldehyde moiety; AA n , dialdehyde with n carbon atoms; A n , aldehyde with n carbon atoms.

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tios of the CGC areas of nonconjugated 18:2 to 18:0 and of 20:2 to 18:0 were calculated before and after addition of 2 μ l of 10% tetramethylammonium hydroxide in methanol and the difference from 100%, divided by 0.8 (the equilibrium ratio), gave percent conjugatable dienes. The needle of the 10- μ l microsyringe was heated 10 sec in the injector port before the tetramethylammonium hydroxide plus sample was injected, at a rate of 0.5 μ l/sec. Percent conjugatable dienes is equal to percent of malondialdehyde in dialdehydes and is more accurately measurable. The conjugatable diene was substituted for malondialdehyde in the matrix-computer calculations.

Computer Solutions. The matrix for aldehyde data from 18:2 isomers was solved on an Apple II computer by a Fortran program (1). A similar matrix was constructed for 20:2 isomers. For 20:3 and 20:4 isomers, solution by matrices was not feasible. 18:2, 20:2, 20:3, and 20:4 fractions were subjected to CGC on a Carbowax free fatty acid phase column programmed from 190°C to 220°C at 2°C/min, using a Packard 428 chromatograph with injection port at 250°C, detector at 280°C, and He split ratio of 100:1.

RESULTS AND DISCUSSION

Octadecenoate Isomers in the Dietary Fats. Octadecenoate isomers were measured by ozonolysis. Oleic acid, 9-18:1, was the only 18:1 isomer in corn oil. The EFAD diet contained fully saturated hydrogenated coconut oil and hence no 18:1. The 18:1 acids of PHSO measured by ozonolysis of the 18:1 fraction ranged from 5-18:1 to 15-18:1 in a Gaussian distribution about carbon atom positions 9 and 10. *cis* and *trans* isomers occur for each positional isomer theoretically in ratios of 1:2. *cis* and *trans* isomers are desaturated by liver microsomes to form mixed *cis*- and *trans*-18:2 (2).

Unsaturated FA Compositions of Liver PL. Compositions are given in Table 1. Of the 18:1 isomers in liver PL of rats fed corn oil, 9-18:1 was the most abundant, with slightly less 11-18:1, derived by elongation of 9-16:1. The 7- and 8-isomers were also present. In liver PL of EFAD, the 18:1 isomers ranged from 5- to 13-18:1, and 10-18:1 of liver PL was nearly as abundant as 9-18:1. In PL from PHSO-fed rats, isomers ranged from 5-18:1 to 14-18:1; 9-18:1 was major, with much less 11-18:1.

Octadecadienoate Isomers. CGC analyses were performed on the 18:2 FA from liver PL from animals of each group. In rats fed corn oil, the 18:2 was 11% of total unsaturated FA of PL (Table 1), and it was largely one component. The 18:2 from liver PL of rats fed EFAD diet was 1% of the total FA of PL, and it showed three major peaks on both CGC columns. From the PHSO-fed rats, the 18:2 fraction was 3% of the FA of PL, and four components were seen on both CGC columns. The isomerism of 18:2 was more complex in EFAD- and PHSO-fed groups than in the corn oil group, and some uncommon 18:2 isomers were detectable.

Histograms of aldehydic ozonolysis products of 18:2 from corn oil-fed rats (data not shown) had the simplest pattern, consisting of AE9, AA3, and A6, confirming the structure of 9,12-18:2. The 18:2 fraction from EFAD rats gave prominent AE5, AE6, AE8, and AE9 products, AA3, and more A7 than A6. PHSO-fed rats gave products ranging from AE5 to AE9,

AA3, and AA4 and decreasing amounts of A6 to A9. Thus, the complexity of 18:2 isomers in EFAD- and PHSO-fed rats was confirmed.

Data from aldehyde analyses of liver PL from each of five rats chosen randomly from each of the three diet groups were entered into data files. For each sample, each 18:2 isomer present was identified and quantified by linear simultaneous equations relating the aldehyde, dialdehyde, and aldehyde ester data (1). Histograms of each of the isomers of 18:2 identified and measured in the liver PL for each of five rats of each diet group are shown in Fig. 1 in a pseudo-three-dimensional computer-programmed plot. In corn oil-fed control rats, 18:2 was almost completely 9,12-18:2, linoleic acid. In EFAD rats, 9,12-18:2 was considerably diminished and replaced by 8,11-18:2 and small but consistent proportions of 5,9-18:2. In PHSO-fed rats, 9,12-18:2 was suppressed significantly and replaced by isomers of variable structures and nonuniform occurrence. The 5,9-18:2 and 8,11-18:2 were most consistently present in small proportions, and 5,11-18:2, 5,12-18:2, 6,10-18:2, and 6,11-18:2 each occurred in two rats of the PHSO group. EFAD required that the animal make substitutions of isomeric 18:2 FA endogenously produced, and in PHSO 18:2 isomers were formed from uncommon isomeric monoenoic dietary precursors. The most likely metabolic origin of each isomer was deduced from known desaturations by liver microsomes (2).

In 1965, a time study of replacement of linoleic acid by uncommon isomers of 16:1, 18:1, and 18:2 in lipids of EFA-deficient rats was made by Sand *et al.* (6, 11). With onset of EFAD 18:2 decreased, and 16:1 (identified as 9-16:1) and 18:1 (largely 9-18:1) increased in proportion. In our study of liver PL in EFAD, total 18:1 increased 2.7-fold and 10-18:1 increased in proportion, whereas 11-18:1 decreased within 18:1. Sand *et al.* reported that linoleic acid (9,12-18:2) was the major 18:2 isomer in early EFAD, diminishing sharply as deficiency developed, and the 8,11-18:2, 6,9-18:2 and 5,8-18:2 isomers were elevated in proportion. Our study revealed a uniform occurrence of 8,11-18:2, no 6,9-18:2 present, and a uniform occurrence of a small proportion of 5,9-18:2. The techniques available in 1965 could not have detected 5,9-18:2, and it would have been deduced as 5,8-18:2 from the C₉ aldehyde. At that time, it was assumed that all dienoic acids were methylene interrupted.

The 18:2 isomers found in rat liver PL by computer analysis can be explained by rates of desaturation measured upon individual 18:1 isomeric substrates (2-4) and by the presence or absence of the 18:1 isomers in the dietary fat. CO provided only oleic acid, the naturally occurring 9-18:1 isomer, so the other trace isomers of 18:1 found in liver PL must be of endogenous origin. EFAD diet provided no 18:1 acids, and isomeric 18:1 of liver PL therefore must be of endogenous origin. PHSO provided 18:1 isomers, and 5,11-, 5,12-, 6,11-, and 6,12-18:2 isomers found in liver PL of PHSO-fed rats could have arisen by Δ 5 desaturation and Δ 6 desaturation of 11-18:1 and 12-18:1.

The 18:2 Fraction Isolated from PHSO. This fraction has been shown to consist of several isomers (12). The 18:2 fraction isolated from PHSO used in this study was ozonized, and its fragments indicated a diversity of isomers in the traces of 18:2 present. Aldehyde ester, indicating position of the

Table 1. Unsaturated FA groups in liver PL of rats fed corn oil, EFAD diet, or PHSO

Diet	n	% of total FA in liver PL				
		18:1	18:2	20:2	20:3	20:4
Corn oil	6	6.2 $\pm$ 0.4	10.8 $\pm$ 1.5	0.4 $\pm$ 0.4	1.0 $\pm$ 0.4	27.8 $\pm$ 1.1
EFAD	6	17.1 $\pm$ 1.7	0.8 $\pm$ 0.1	0.3 $\pm$ 0.1	17.7 $\pm$ 1.2	6.2 $\pm$ 1.1
PHSO	8	28.0 $\pm$ 2.0	2.6 $\pm$ 0.2	0.4 $\pm$ 0.1	9.4 $\pm$ 0.8	7.4 $\pm$ 0.3

Values are mean $\pm$ SD.

FIG. 1. Isomeric 18:2 FA in liver PL, identified and measured by computer solution of simultaneous equations, using data from quantified ozonolysis of each of five samples (horizontal lines) from each dietary group, expressed as percent of total 18:2.

double bond closest to the carboxyl group, ranged from C₅ to C₁₄. Aldehydes, which indicate position of the double bond nearest the methyl group, ranged from C₃ to C₇. Conjugatability of dienoate double bonds (11.2%) showed that most 18:2 isomers present in PHSO had isolated double bonds separated by more than 3 carbon atoms. In contrast, most isomers found in liver PL of rats fed EFAD diet were methylene interrupted, as is true for all known EFA. In EFAD, 9-16:1 becomes a dominant monoenoic acid (11). Subsequent Δ 6 desaturation could yield 6,9-16:2 and elongation could yield 8,11-18:2, the major 18:2 isomer in liver PL of our EFAD rats.

Eicosadienoate Isomers. The 20:2 fraction isolated from rat liver PL by HPLC was analyzed as described above for 18:2 isomers. CGC analyses of the FA from liver PL of rats fed corn oil showed that the 20:2 fraction was 0.35% of FA of PL (Table 1) and it was largely a single component. The 20:2 from liver PL of EFAD rats was 0.34%, and it showed two large multicomponent peaks and two smaller peaks. From PHSO-fed rats, the 20:2 was 0.43% of FA of PL, and only two peaks were separated, consisting of several overlapping components. Comparable inseparability of pure synthetic 18:2 iso-

mers by CGC has been demonstrated (1). The principal component of 20:2 from corn oil-fed rats was 11,14-20:2, metabolically derived by chain elongation of 9,12-18:2 (13).

Histograms for aldehyde ester, dialdehyde, and aldehyde from the 20:2 fractions of liver PL from the three groups were compared (data not shown). The 20:2 fraction from corn oil-fed rats had a simple pattern consisting essentially of AE11, AA3, and A6 fragments, confirming the structure to be 11,14-20:2. EFAD rats had prominent AE8, AE10, AA3, AA4, A7, and A9 fragments. By contrast, PHSO rats had aldehyde ester homologs ranging from C₅ to C₁₀, dialdehyde homologs from C₃ to C₆, and aldehyde homologs from C₆ to C₉.

The aldehyde compositions for each of four rats chosen randomly from the three groups were entered into the appropriate matrices and solved by computer for each possible 20:2 isomer. The contents of all observed 20:2 isomers in the liver PL of rats from each group are arranged in Fig. 2. The 20:2 from corn oil-fed rats was almost all 11,14-20:2, as might be expected from 2-carbon extension of 9,12-18:2 (14). In EFAD, 10,13-, 8,11-, 8,13-, 10,14-, and 7,11-20:2 were found, in order of decreasing abundance. In PHSO-fed rats, 8,14-, 6,11-, 10,13-, and 7,11-20:2 isomers were found.

FIG. 2. Isomeric 20:2 FA in liver PL, identified and measured by computer solution of simultaneous equations, using data from quantified ozonolysis of each of four samples from each dietary group, expressed as percent of total 20:2.

Most of the 20:2 isomers observed are explained by 2-carbon elongation of the 18:2 isomers present. 9,12-18:2 occurred in all three groups, but only in the CO group, in which 9,12-18:2 was abundant, was it elongated. In EFAD and PHSO-fed rats, the 9,12-18:2 was conserved for reactions essential to the animal, and it was not converted to the dead-end side product of the ω 6 cascade. Neither precursor nor product of the reactions $5,9-18:2 \rightarrow 7,11-20:2$ and $8,11-18:2 \rightarrow 10,13-20:2$ occurred when linoleic acid was adequate, but both occurred in EFAD and PHSO feeding, indicating that 9,12-18:2 was inadequate. The 6,9-18:2 isomer occurred in EFAD liver and was elongated to 8,11-20:2. The 5,11-, 5,12-, and 6,11-18:2 isomers, which occurred in some rats fed PHSO, were not elongated to corresponding 20:2 isomers. The 10,14-20:2 isomer occurred strongly in PHSO liver and as a minor component in corn oil liver, but its shorter-chain homolog, 8,12-18:2, did not. One explanation may be that 8,12-18:2 may be formed but not incorporated into PL, whereas it may be elongated to 10,14-20:2 and inserted into PL.

Eicosatrienoate and Eicosatetraenoate Isomers. CGC analysis indicated that the presence of unusual isomers observed in 18:1, 18:2, and 20:2 fractions in liver PL in EFAD- and PHSO-fed rats continued in the 20:3 and 20:4 fractions. A typical CGC analysis from each group is shown in Fig. 3. Concentrates of 20:3 and 20:4 were isolated by AgNO_3 TLC and purified by HPLC on a reverse-phase column. When these components were ozonized, reduced, and subjected to CGC on an SP-2330 column, the aldehyde and aldehyde ester fragments could be identified and measured.

Total 20:3 was lowest in the liver PL of corn oil-fed rats, 0.97% compared to the 17.7% in EFAD rats and 9.4% in PHSO rats (Table 1). Three peaks were evident for 20:3 isomers in corn oil-fed rats, and the largest of these must be 8,11,14-20:3 (20:3 ω 6). In EFAD, the major 20:3 isomer is known to be 5,8,11-20:3 (20:3 ω 9), and this was confirmed by HPLC isolation, ozonization, and CGC. Only AE5 and A9 were produced from this 20:3 isomer, confirming it to be Mead's acid, 5,8,11-20:3. A minor isomer, probably 20:3 ω 6, and several others of unknown structure also occurred. In PHSO rats, as many as six isomers of 20:3 were evident, largely 5,8,11-20:3.

The 20:4 from the corn oil livers gave only A6 and AE5, confirming it to be arachidonic acid, 5,8,11,14-20:4. The 20:4 from EFAD rat liver produced largely A5 and A6, indicating that some arachidonic acid was present despite the EFAD. From PHSO-fed rats, the major 20:4 isomer was 5,8,11,14-20:4.

The single 20:4 component of liver PL in corn oil-fed rats was 27.8%, compared to 6.2% total 20:4 in EFAD rats and 7.4% total 20:4 in PHSO rats, confirming our report of suppression of arachidonate by dietary PHSO (15). CGC analysis of FA of liver PL in EFAD showed one major and one minor isomer, probably 20:4 ω 6 and 20:4 ω 7. PHSO rats showed one major and two minor isomers of 20:4. Suppression of 20:4 ω 6 by EFAD diet was evident, and PHSO also suppressed 20:4 to a low value despite the presumed presence of 9,12-18:2 in PHSO (15), and the 20:4 consisted of three components, of which 20:4 ω 6 was one. The multiplicity of isomeric PUFA at each step in the pathway from 18:2 ω 6 to 20:4 ω 6 may contribute to diminished essential isomers in membranes (15), the precursors of eicosanoids.

Earlier studies showed that PHSO (15, 16) or concentrates of *cis*- and *trans*-18:1 isomers from PHSO (3) suppress 20:4 ω 6 in tissue lipids. Studies with liver microsomes and the complete series of labeled positional *cis*- and *trans*-18:1 isomers revealed that microsomes desaturate many of these to unusual isomers of 18:2 (2-4), leading to the hypothesis that unusual isomers of 18:2, 20:2, 20:3, and 20:4 may exist in tissues of PHSO-fed animals, and they may be precursors of unusual eicosanoids or inhibit synthesis of normal eicosanoids (2). This report presents analytical evidence that multiple isomers did indeed occur in the 18:2, 20:2, 20:3, and 20:4 fractions of FA of rat liver PL when PHSO was fed. Unusual isomers at each step of the ω 6 metabolic cascade must therefore be considered as possible inhibitors of subsequent steps in the cascade leading to diminished 20:4 ω 6 synthesis when PHSO is fed.

A study of the unnatural all-*cis* dienoic acids, 9,15-18:2, 12,15-18:2, and 7,13-20:2, showed that all were incorporated into liver PL (17). None were desaturated or elongated to longer-chain PUFA. The distributions of these isomers in the α and β positions of PL differed. Feeding 9,15-18:2 or 7,13-20:2 suppressed the synthesis of arachidonic acid and 22:5 ω 6. Clearly, some unnatural isomers distort PUFA metabolism. Acyltransferase activity upon isomeric *cis*, *cis* methylene-interrupted 18:2 substrates showed considerable specificity for individual isomers in positions 1 and 2 of phosphatidylcholine (18). The natural 9,12 isomer was rapidly esterified at position 2 but not at position 1. In contrast, six other isomers were rapidly esterified at position 1. The alternating activities of 8,11, 9,12, and 10,13 isomers and their inverse effects at positions 1 and 2 indicate that structure near carbon atoms 8-10 is critical in the metabolism of PUFA. Clearly, shift of a double bond by one carbon atom directs the metabolism of a PUFA into different pathways.

FIG. 3. Segments of typical capillary gas chromatograms of single rat livers from each dietary group (CO, corn oil), showing the 20:3 and 20:4 regions, revealing the effect of dietary fat upon complexity of isomerism of 20:3 and 20:4.

Although a recent study did not discuss positional isomers present in hydrogenated fat, “*trans*” isomers of 18:1 were found to be “at least as unfavorable as that of the cholesterol-raising saturated fatty acids, because they not only raise LDL cholesterol levels but also lower HDL cholesterol levels” (19). That study involved both *cis* and positional isomers as well as the *trans* isomers, which were emphasized. Therefore, the unusual positional isomers discussed here probably contributed to the effects upon cholesterol transport observed.

It is now clear that uncommon isomers of PUFA occur in the lipids of animals fed partially hydrogenated fat and that they inhibit the metabolism of PUFA at many steps in the normal metabolic cascade. It would, therefore, seem wise to avoid foods that contain unusual or unnatural isomeric PUFA or their isomeric monoenoic FA precursors. The latter, both *cis* and *trans* positional isomers of 18:1, occur abundantly in partially hydrogenated vegetable oils now commonly consumed by Western populations. The large-scale hydrogenation of vegetable oils reduces ω 3 and ω 6 EFA and replaces them by saturated and isomeric 18:1 acids that interfere with the ω 3 and ω 6 metabolism, inducing significant partial deficiencies of EFA. It would seem wise to preserve the essential nutrients and to avoid producing inhibitors of their metabolism by hydrogenation. Evidence is growing for the essentiality of ω 3 PUFA and the occurrence of deficiencies of ω 3 acids in humans under stress conditions (20). It would, therefore, be wise economy to use oils containing linolenic acid directly as foods and to avoid their hydrogenation.

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