Hydrogenation of a Menhaden Oil: II. Formation and Evolution of the C_{20} Dienoic and Trienoic Fatty Acids as a Function of the Degree of Hydrogenation[1]

J-L. SEBEDIO[2] and R.G. ACKMAN, Canadian Institute of Fisheries Technology, Technical University of Nova Scotia, PO Box 1000, Halifax, Nova Scotia, Canada B3J 2X4

ABSTRACT

To complement studies on monoethylenic fatty acids produced from the major polyunsaturated fatty acid (20:5Δ5,8,11,14,17) during hydrogenation of a menhaden oil of iodine value (IV) 159, the C_{20} dienoic and trienoic fatty acid isomers of partially hydrogenated menhaden oils (PHMO) of IV 131.5, 96.5 and 85.5 were isolated by a combination of preparative gas liquid chromatography (GLC), mercuric adduct fractionation, and silver nitrate thin layer chromatography ($AgNO_3$-TLC). The 20:2 fatty acid methyl esters of the three PHMO samples were transformed to the corresponding alcohols and ozonized in BF_3-MeOH, followed by GLC analysis of the ozonolysis fragments. During the hydrogenation process, residual ethylenic bonds in the 20:2 isomers tend to migrate both towards the carboxyl group and towards the methyl end of the molecule. The hydrazine reaction results revealed that the *trans* ethylenic bonds in the 20:2 and 20:3 isomers were distributed all along the the carbon chain, but the *cis* ethylenic bonds were more localized in the Δ11,Δ14 and Δ17 positions of the preexisting major menhaden oil component 20:5Δ5,8,11,14,17. Iatroscan analyses on $AgNO_3$-chromarods revealed that, as a result of the hydrogenation process, almost half of the 20:2 isomers were non-methylene-interrupted *cis, trans/trans, cis* structures.

INTRODUCTION

The structures of monoethylenic isomers of both partially hydrogenated marine oils (1-4) and vegetable oils (5-8) have been determined in detail. However, the only structural studies of dienoic and trienoic fatty acids in edible oils have been carried out on partially hydrogenated vegetable oils or methyl esters (9-14). These studies have generally used a combination of gas liquid chromatography (GLC) and reductive ozonolysis (15). A computer method (16) was also developed to analyze the different fragments resulting from the ozonolysis of a mixture of dienoic fatty acids in order to give the distribution of isomers. Although dienoic and trienoic fatty acids of partially hydrogenated marine oils represent a large proportion of the total fatty acids (17,18), they have not been studied. Knowledge of their structures is a prerequisite to further studies on the metabolism of partially hydrogenated marine oils in rats, pigs or nonhuman primates (19-21). The study of their structures also contributes to a better understanding of the hydrogenation process, the monoethylenic isomer composition (17), and the reactivity of the ethylenic bonds of the very highly unsaturated fatty acids (20:5, 22:6) typically found in marine oils.

MATERIALS AND METHODS

The partially hydrogenated menhaden oils used for this study were from the series prepared for the determination of the C_{20} monoethylenic fatty acids (17). Each sample was saponified (AOCS Method Ca 6b-53), the unsaponifiables removed and the recovered fatty acids converted to methyl esters by refluxing in a solution of 7% BF_3 in MeOH for 15 min (22). A nitrogen atmosphere was maintained at all times.

Isolation of 20:2 and 20:3 Fatty Acid Classes

The 20:2 and 20:3 fatty acid methyl esters of three partially hydrogenated menhaden oils (IV 131.5, 96.5 and 84.5) were isolated by preparative gas liquid chromatography to collect the C_{20} acids from an SE-30 column (23), followed by thin layer chromatography (TLC) fractionation of the methoxy-bromomercuri adducts using dioxane/hexane (40:60) as the solvent system (24). Each TLC band was then treated with HCl (25) and the recovered methyl esters extracted with chloroform.

Gas Liquid Chromatography

All analytical gas liquid chromatography analyses were performed as described elsewhere (4).

Hydrazine Reduction of Eicosadienoic and Eicosatrienoic Fatty Acid Methyl Ester Isomers

A few milligrams (1-5 mg) of the isolated 20:2 and 20:3 methyl ester isomers were submitted to the action of hydrazine in ethanol in the presence of oxygen (26-28). Following the addition of H_2O, the fatty acid methyl esters were extracted with diethyl ether. The ether layer was dried and the resulting mixture of fatty acids was fractionated by TLC of the methoxy-bromomercuri adducts (24). The monoenes were recovered from the TLC band, regenerated and further fractionated into *cis* and *trans* isomers by silver nitrate TLC (29).

Ozonolysis of Dienoic Fatty Acid Isomers in BF_3/MeOH, and Analysis of Products

The dienoic fatty acid methyl esters were reduced to the corresponding alcohol by reaction with the Vitride reagent (30). The ozonolysis was then carried out on the alcohols by the method developed for methyl esters (31). The resulting fragments, monomethyl esters, alcohol esters and diesters were fractionated by TLC (Prekotes; Adsorbosil-5, Applied Science Laboratories) using hexane/diethyl ether/acetic acid (80:20:1) as the solvent system. Three TCL bands were obtained; alcohol esters (R_f=0.07), dimethyl esters (R_f=0.26) and monomethyl esters (R_f=0.56). Each band was extracted three times with chloroform to maximize the recovery of the short-chain mono- and diesters. The mono- and diesters were analyzed directly by GLC on a BDS (butanediol succinate polyester) wall-coated open-tubular column, the monoesters at 90 C and the diesters at 100 C and 160 C. The alcohol esters were transformed to the acetate derivatives by reaction with acetic anhydride prior to the GLC analysis. The resulting acetate esters were analyzed on a BDS column at 170 C.

Ozonolysis of Monounsaturated Fatty Acid Methyl Esters

Ozonolysis of monounsaturated fatty acid methyl esters was effected in a 7% BF_3/MeOH solution as originally described (32). The resulting fragments (monoesters and diesters), extracted with chloroform (31), were analyzed on a BDS column. Quantitation was based on diester analyses carried out isothermally at 100 C and 160 C to cover the wide range of products.

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[2]Present address INRA, Aliments de l'homme, 17 rue Sully, 21034 Dijon, France.

Study of C_{20} Dienoic Fatty Acid Isomers with the $AgNO_3$-Chromarod TLC/FID System (Iatroscan)

Chromarods-S impregnated with silver nitrate, as described for monoethylenic acids (33), were used for the study of dienoic fatty acid siomers. The rods were developed for 25 min in pure benzene and the quantitative analyses based on averages of recorder peaks from 10 rods.

RESULTS AND DISCUSSION

The GLC analyses of the isolated 20:2 fractions on SILAR-7CP (Fig. 1) of the initial and the three partially hydrogenated oils showed that, as the total 20:2 content in the oil increased (17), many new isomers were formed. The appearance of an early eluting group of 20:2 isomers with retention times identical to some 20:1 isomers, even with the modest reduction in IV from 159.0 to 131.5, seemed to indicate the formation of an important quantity of all-*trans* dienoic isomers which would elute earlier than the *cis* isomers on SILAR-7CP (34), as well as of NMID (nonmethylene-interruped dienoic) acids generally, which also elute earlier than MID (methylene-interruped dienoic) acids (33).

To study the relationship between the position of the ethylenic bonds and the GLC retention time, the total 20:2 fraction of each sample was transformed to the corresponding alcohols which were then subjected to oxidative ozonolysis as described for C_{18} dienoic acids (30). The three different fragments from the ozonolysis of a dienoic alcohol identify the bond positions:

$$CH_3\text{-}(CH_2)_X\text{-}CH{=}CH\text{-}(CH_2)_Y\text{-}CH{=}CH\text{-}(CH_2)_Z\text{-}CO_2CH_3$$

$$\downarrow \text{ (1) Vitride; (2) } O_3,\ BF_3/CH_3OH$$

$$CH_3\text{-}(CH_2)_X\text{-}CO_2CH_3$$
$$+\ CH_3O_2C\text{-}(CH_2)_Y\text{-}CO_2CH_3$$
$$+\ CH_3O_2C\text{-}(CH_2)_Z\text{-}CH_2OH$$

$$\downarrow (CH_3\text{-}CO)_2O$$

$$CH_3O_2C\text{-}(CH_2)_Z\text{-}CH_2OCOCH_3$$

The GLC analysis of the monoester fragment gives the position of the ethylenic bond closest to the methyl end of the molecule. This is an analytical problem requiring further study as C_3, C_4 and C_5 are difficult to recover if present. These GLC analyses for monoesters with more than five carbon atoms were corrected for both the extraction procedure, and the flame ionization (FID) response factor, with the data presented in Table I. The only large FID response factor so obtained was for the hexanoic acid methyl ester (1.13).

From the other two fragments (diester and acetate ester), the position of the ethylenic bond closest to the carboxyl group can be determined. The GLC analyses of the diester fragments were also corrected for both the extraction procedure and the flame ionization response factor (35). However, under the GLC conditions used, it was not practical to correct the acetate ester data for the FID response factor (36) due to a partial decomposition of these compounds with higher molecular weights during the GLC analysis. This novel problem is still under review but does not prevent generalities being deduced from the uncorrected proportions.

Most of C_{20} dienoic isomers are formed from the progressive (stepwise) hydrogenation of 20:5Δ5,8,11,14,17

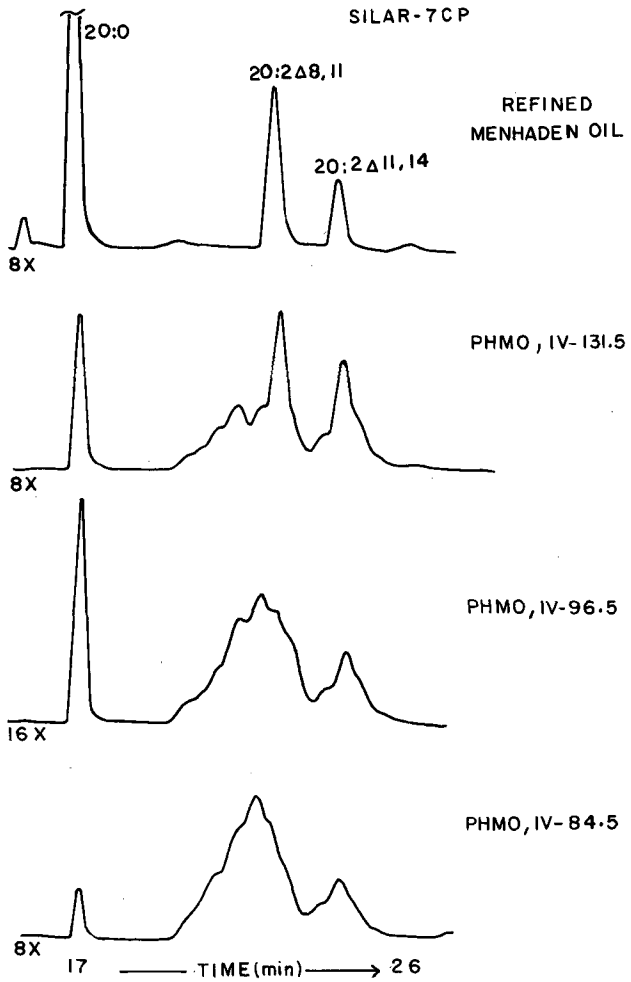

FIG. 1. GLC analyses of the isolated C_{20} dienes of a refined and three partially hydrogenated menhaden oils, with 20:0 as internal standard. Column; wall-coated stainless steel, 47 m × 0.25 mm id, liquid phase SILAR-7CP, operated at 160 C.

TABLE I

Recovery (%) of Short-Chain Monoesters by Extraction with $CHCl_3$ and Flame Ionization Correction Factors (FID) for the GLC Analysis (BDS) Monoesters (relative to methyl stearate as 1.00)

Monoester	Recovery (wt %)	FID correction factor
C_6	74.5	1.13
C_7	87.7	1.06
C_8	98.5	1.05
C_9	91.5	1.04
C_{10}	96.0	1.04
C_{11}	98.0	1.03
C_{12}	99.2	1.03

and not by geometrical and positional isomerization of the preexisting 20:2 (originally 0.6% of the oil of IV 159.0, compared to a total 20:2 content of 6.6% at IV 84.5 (17). The ozonolysis results, especially for the acetate esters (Fig. 2), indicate that positional isomerization is taking place concurrently with the progressive hydrogenation of the highly unsaturated fatty acid, in this case 20:5ω3. The most favored position of the residual ethylenic bond closest to the carboxyl group down to an iodine value of 84.5 is Δ5, and the Δ8 isomer is clearly an intermediate reflecting the parent bond positions before a progressive delocalization of the intermediate ethylenic bonds occurs when the iodine value is decreased from 131.5 to 84.5.

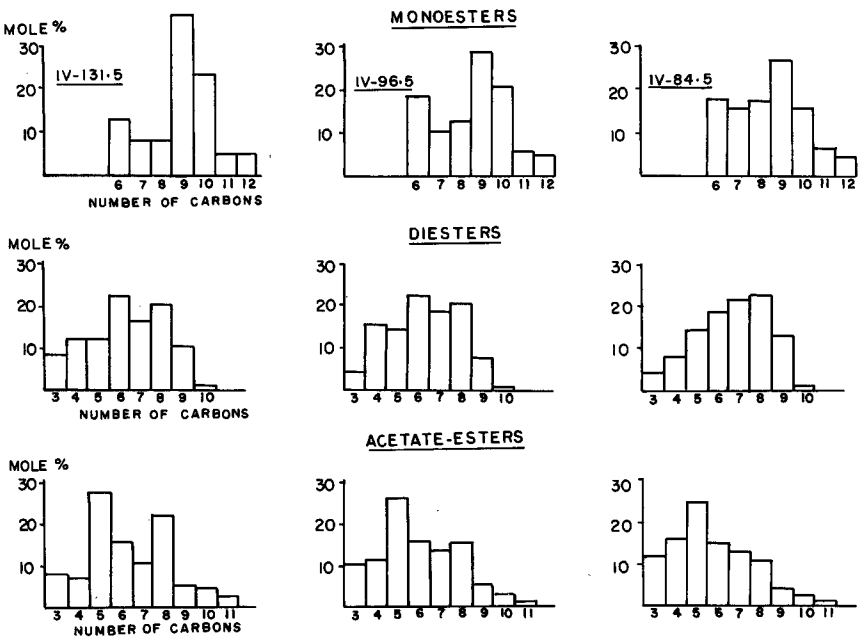

FIG. 2. Ozonolysis products (mole %) of the total 20:2 alcohols from partially hydrogenated menhaden oils of IV of 131.5, 96.5 and 84.5. Results included recovery and FID correction factors for mono- and diesters but not for the alcohol-acetate esters.

A close examination of the three classes of fragments for the three samples of partially hydrogenated oils shows that in the 20:2 dienes, one ethylenic bond tends to migrate towards the carboxyl group as the proportion of acetate esters with shorter chains increases at lower iodine values. The other ethylenic bond tends to migrate towards the methyl end of the molecule as greater proportions of C_6-C_8 monoesters are found in the partially hydrogenated oils of lower iodine values. Confirmation of this divergency phenomenon is given by the examination of the diester fragments (Fig. 2) where the shortest middle fragments, dimethyl malonate (DMC_3), and succinate (DMC_4) decrease in favor of longer chain lengths as the IV is decreasing. Retention of some bond positions from 20:5Δ5, 8,11,14,17 gives an intermediate C_6 diester. This migration of the ethylenic bond towards the methyl end of the molecule has been already observed in the study of the 20:1 isomers (17) where at IV 84.5 there were more *trans* isomers with the ethylenic bond position Δ12-Δ17 than in position Δ4-Δ10. Once four or more methylene groups separate the two ethylenic bonds, the one remote from the carboxyl group is evidently functionally independent of the one nearer the carboxyl group.

The ozonolysis of the 20:1 isomers (Table II) produced by the hydrazine reduction of the total 20:2 of the partially hydrogenated menhaden oil of IV 84.5 showed that geometrical isomerization is important during the hydrogenation process (21.2% *cis* 20:1 compared to 78.8% *trans* 20:1). It is important to note that the *cis* ethylenic bonds are mainly in the original Δ11,Δ14, and Δ17 positions of the 20:5Δ5,8,11,14,17, but that the *trans* ethylenic bonds are distributed more equitably along the carbon chain. However, great care must be taken with the quantitative interpretation of the hydrazine reduction products of unsaturated fatty acids. It has been shown (28) that three factors mainly govern the reactivity of dienoic fatty acids towards hydrazine: (a) the geometry of the ethylenic bond – *trans* ethylenic bonds react faster than the corresponding *cis* bonds; (b) the position of the ethylenic bond on the carbon chain – ethylenic bonds close to either the carboxyl group or the methyl group react faster than those located in the center of the molecule. Note, for example, the low proportion of Δ5 and Δ17 isomers of 20:1 illustrated as products from 20:5ω3 (24,23); and (c) the relative position of the two ethylenic bonds – the methylene-interrupted dienes react faster than the nonmethylene-interrupted dienes.

TABLE II

Totals and Distributions of *cis* and *trans* 20:1 Isomers Produced by the Hydrazine Reduction of the 20:2 Fatty Acid Methyl Esters of Partially Hydrogenated Menhaden Oil (IV 84.5)

Ethylenic bond position Δ	ω	*cis*	*trans*
Σ%		21.2	78.8
3	17	4.2	3.4
4	16	6.3	6.6
5	15	2.1	7.4
6	14	8.6	13.0
7	13	2.1	8.0
8	12	3.2	8.8
9	11	5.3	9.1
10	10	5.0	4.6
11	9	16.0	4.7
12	8	4.3	4.4
13	7	3.9	4.6
14	6	10.3	6.5
15	5	3.0	6.6
16	4	5.0	6.3
17	3	18.2	4.7
18	2	2.5	1.3

Considering these three factors, it is not surprising to find a different ratio of Δ5/Δ6 in the hydrazine products (Table II) for both the *cis* and the *trans* isomers when compared with the ratios found in the ozonolysis products of the total 20:2 (Fig. 2). One possible explanation of these different Δ5/Δ6 ratios could be a large difference in reactivities of the Δ5 and Δ6 ethylenic bonds for the hydrazine reaction. In this case, it would be necessary to synthesize or isolate these isomers and compare their re-

activities with an acid having one unsaturation in the center of the carbon chain (28). Another possible explanation is the position of the Δ5 or Δ6 ethylenic bond relative to the second unsaturation of the dienoic system. The ozonolysis products (Fig. 2) imply that the Δ5 position is found in an important number of NMID, which hydrogenate with hydrazine more slowly than MID, resulting in the formation of a less than quantitative proportion of 20:1Δ5.

The hydrazine reaction products could also be used for the verification by GLC of the positions of the ethylenic bonds closer to the methyl end of the molecule as no monoester shorter than C_6 could be quantitatively recovered from the ozonolysis of 20:2 isomers (Fig. 2). From the accumulation of some original ethylenic bond positions (remote from the carboxyl group, e.g., *cis* Δ11,Δ14 and Δ17), all of which elute later than isomers with more centrally located positions (2,20,33,37), it can be deduced that the later eluting peak in the chromatogram for IV 84.5 (Fig. 1) is likely to include the *cis* Δ11,Δ14 or Δ17 bonds combined with another *cis* or *trans* bond in certain nearby positions. The accumulation of *trans* Δ6 (Table II), which in monoethylenic from has one of the shortest retention times on polyester GLC phases (37), will lead to a variety of 20:2 isomers represented by the earlier eluting material (Fig. 1).

A good separation of the positional and geometrical 20:2 isomers into various classes was obtained on silver nitrate-impregnated chromarods using benzene as solvent (Fig. 3); the *cis,cis* nonmethylene-interruped dienes migrated the least, then the *cis,cis* methylene-interrupted dienes mixed with the *cis,trans* + *trans,cis* NMID. The *trans,trans* NMID together with *cis,trans* + *trans,cis* MID were the most mobile classes found. No *trans,trans* MID was detected. It was apparent (Fig. 1) that only minor amounts of MID remained after reduction at IV 84.5. We propose that for the oil sample of IV 84.5, the two larger peaks (Fig. 3) do not include much of *cis,cis* MID and that the total *cis,trans* + *trans,cis* MID is exceeded by the *trans, trans* NMID. The quantitative results (Table III) show that the dienes seem to be stabilized with one ethylenic bond in a *cis* configuration and the other one is a *trans* configuration (44.7% *c,t* + *t,c* NMID). The sharpness of the peak in Figure 3 suggests that an important quantity of *trans,trans* NMID was also formed at IV 84.5 (Table III). These isomers could be included in the early eluting GLC peak of Figure 1.

The *cis* and *trans* 20:1 isomers formed by the hydrazine reduction of the total 20:3 isomers were similar to those observed for the hydrazine reduction of the total 20:2 isomers. The *cis* isomers (Table IV) were localized in the Δ11,Δ14 and Δ17 positions but not notably in the Δ8 position. However a large proportion of artifact Δ4,Δ6 and Δ9 ethylenic bonds were also formed. The *trans* ethylenic bonds (Table IV) were distributed all along the carbon chain with a preferential accumulation of the isomers close to the carboxyl group.

The major difference in the 20:2 and 20:3 fatty acids (Tables II and IV) is the amount of *cis* 20:1Δ18 obtained after the hydrazine reduction (none for the 20:3 compared to 2.5% for the 20:2). This result could mean that the original 20:5ω3 mostly stays on the catalyst with a strong bonding of the Δ11,Δ14 and Δ17 bonds until 2 or 3 bonds are reduced. At the same time, some of the *cis* ethylenic bonds are converted to the *trans* isomers, followed by a migration of the *trans* ethylenic bonds along the carbon chain. This mechanism would explain the *trans* isomer compositions (Tables II and IV). The longer the C_{20} polyunsaturated acid stays on the catalyst, the more likely the formation of *cis* Δ18. Hence, 20:2 includes the *cis* Δ18 bond, whereas more 20:3 has "escaped" from the catalyst.

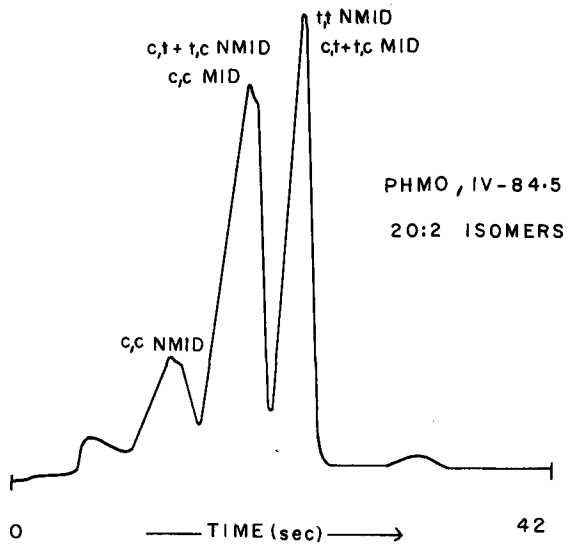

FIG. 3. Iatroscan analysis on silver nitrate-impregnated Chromarods-S of the 20:2 isomers of a partially hydrogenated menhaden oil (IV 84.5). Solvent was benzene.

TABLE III

Isomer Class Composition of the C_{20} Dienoic Fatty Acids of Partially Hydrogenated Menhaden Oil Samples by Silver Nitrate-Impregnated Chromarods-S

Fatty acid	Partially hydrogenated oils IV 131.5	IV 96.5	IV 84.5
cis,cis NMID	23.4 ± 3.0	21.7 ± 1.5	20.4 ± 1.5
cis,trans + *trans,cis* NMID + *cis,cis* MID	67.2 ± 3.0	48.4 ± 2.9	44.7 ± 0.7
trans,trans NMID + *cis,trans* + *trans,cis* MID	9.4 ± 1.1	29.9 ± 2.9	34.9 ± 1.9

TABLE IV

Totals and Distributions of *cis* and *trans* 20:1 Isomers Produced by the Hydrazine Reduction of the 20:3 Fatty Acid Methyl Esters of Partially Hydrogenated Menhaden Oil (IV 84.5)

Ethylenic bond position Δ	ω	*cis*	*trans*
Σ%		31.7	68.3
3	17	1.6	1.9
4	16	8.2	9.9
5	15	3.8	9.2
6	14	9.8	12.2
7	13	2.6	5.3
8	12	7.2	9.2
9	11	8.5	9.9
10	10	4.9	6.6
11	9	16.5	6.7
12	8	4.7	4.4
13	7	6.1	5.1
14	6	8.6	3.1
15	5	3.2	3.2
16	4	1.4	4.0
17	3	12.0	6.0
18	2	ND[a]	3.3

[a]ND: Not detected under analytical conditions.

The accumulation of the residual 20:2 and 20:3 isomers with *trans* ethylenic bonds close to the carboxyl group tends to verify the results observed through the study of the C_{20} monoethylenic fatty acids (17), where an accumulation of isomers with low numbers was observed

from an IV of 159.0 to 96.5. This would indicate a preferential hydrogenation of *trans* ethylenic bonds far away from the carboxyl group.

The detailed isomer compositions of the 20:1, 20:2 and 20:3 fatty acids indicate that many processes are occurring simultaneously during the hydrogenation of marine oils on nickel catalyst. An important geometrical isomerization as well as an important positional isomerization for *trans* ethylenic bonds is taking place during the hydrogenation process. However the *cis* ethylenic bonds tend to stay in the original Δ11,Δ14 and Δ17 positions of the parent 20:5Δ5,8,11,14,17. The positional isomerization occurs towards both the carboxyl group and the methyl end of the fatty acid molecule.

Although there is a very large literature which discusses the biochemistry (19-21, 38-39) and effects of long-chain (i.e., C_{20} and C_{22}) monoethylenic fatty acids on the hearts of rats, very little has been recorded for the corresponding artifact diethylenic fatty acids. It is known that they can contribute to cardiac lipidosis in the rat (40), and in the cynomolgus monkey fed partially hydrogenated herring oil, the percentage of these complex mistures of non-methylene-interrupted fatty acids in the depot fat is about half (1.2-1.5%) of the percentage (2.9%) in the dietary fat (19). The 20:2 structures elucidated in this report, especially those with well separated monoethylenic bonds, need to be considered in the context of the known biochemistry of long-chain monoethylenic fatty acids, including the recently discovered and novel role of peroxisomes (21). This complex topic (38,39) is beyond the scope of this report which, however, strongly suggests that no one C_{20} dienoic (or trienoic) acid, originating in 20:5Δ5,8,11, 14,17, seems to accumulate in proportions suggestive of a specific role in animal biochemistry.

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