Lipolysis of menhaden oil triacylglycerols and the corresponding fatty acid alkyl esters by pancreatic lipase in vitro: a reexamination

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Abstract In order to distinguish between possible fatty acid differences during lumenal lipolysis and cellular absorption, we have reinvestigated the in vitro hydrolysis of menhaden oil and its alkyl esters by pancreatic lipase. For this purpose we incubated menhaden oil or its fatty acid methyl and ethyl esters with porcine pancreatic lipase in the presence of bile salts and determined the composition of the released free fatty acids, monoacylglycerols, diacylglycerols, and residual triacylglycerols, or the free fatty acids and residual alkyl esters, respectively, by thin-layer and gas–liquid chromatography. There was significant discrimination against the Δ^4- to Δ^7-unsaturated fatty acids of both medium and long chain lengths during the hydrolysis of menhaden oil and its fatty acid ethyl esters. In general, the ethyl esters were hydrolyzed 10–50 times more slowly than the corresponding glyceryl esters, depending on the exact ratio of the two substrate types. None of the triacylglycerols or ethyl esters, however, was completely resistant to hydrolysis resulting in an eventual cleavage of all the alkyl esters and presumably all the primary ester bonds in the triacylglycerol molecules. Since the rate of release of the least resistant fatty acid exceeded that of the most resistant acid by only a factor of 6, it is concluded that in the presence of a large excess of lipase the liberated fatty acids would approach the composition of the dietary alkyl or glyceryl esters, as observed during lumenal lipolysis (Yang, L-Y., A. Kuksis, and J. J. Myher. 1989. *Biochem. Cell Biol.* **67:** 192–204). **—Yang, L-Y., A. Kuksis, and J. J. Myher.** Lipolysis of menhaden oil triacylglycerols and the corresponding fatty acid alkyl esters by pancreatic lipase in vitro: a reexamination. *J. Lipid Res.* 1990. **31:** 137–148.

Supplementary key words fatty acid ethyl esters • monoacylglycerols • molecular species of diacylglycerols • carbon numbers of triacylglycerols • gas–liquid chromatography • thin-layer chromatography

There is much current interest in the beneficial physiological effects of dietary marine oils and their polyunsaturated long chain fatty acid esters (1), but the relative efficiency of digestion and absorption of the various preparations has not been directly determined. On the basis of previous work in vitro (2–4) it has been assumed that the polyunsaturated long chain fatty acid esters would be relatively resistant to pancreatic lipolysis and that this effect would lead to decreased absorption and recoveries of these fatty acids in the circulation. In fact, the apparently lower recoveries of polyunsaturated fatty acids in lymph (5) and plasma (6, 7) have been correlated with the decreased rates of hydrolysis of these esters anticipated from work in vitro. However, other experiments have suggested that the long chain polyunsaturated fatty acids of marine oils are effectively recovered in the adipose tissues of pigs (8) and rats (9) at least when fed over long periods of time. Likewise, a recent review on the digestion and absorption of marine oils has concluded that the polyunsaturated long chain fatty acids are probably completely absorbed despite a possible lower relative rate of release from the esters by pancreatic lipase (10). When administered in the free form as a single dose of an aqueous emulsion (11), radioactive eicosapentaenoic and oleic acids appeared in the lymph of rats at about the same rate. We have recently shown (12) that lumenal hydrolysis of menhaden oil results in an accumulation in the micellar phase of free fatty acids and monoacylglycerols, which are closely similar in composition to the fatty acids present in the *sn*-1(3)- and 2-positions of the original oil, respectively. Similarly, lumenal hydrolysis of the ethyl esters of menhaden oil fatty acids appeared to result in a representative release of both short and long chain fatty acids.

The present study demonstrates that the relative rates of in vitro hydrolysis of the least and most resistant fatty acids differ only by a factor of 6, which is unlikely to result in significant differences in the free fatty acid composition under rapid and extensive hydrolysis in the lumen, although it clearly leads to a nonrepresentative initial release of fatty acids in vitro.

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl.

EXPERIMENTAL PROCEDURES

Materials

Menhaden oil was provided by Zapata Haynie Corporation, Reedville, VA. Corn oil (Mazola) was obtained from a local grocery. Methyl and ethyl esters of menhaden oil fatty acids were prepared (12, 13) by treating the oil with 1 N sodium methoxide or ethoxide in the corresponding alcohol–toluene 60:4 as previously described. Both oils and esters were purified by TLC and were free of significant amounts (less than 0.01%) of free fatty acids. Platinum oxide was purchased from Matheson Coleman and Bell, East Rutherford, NJ. Sodium taurocholate was obtained from Sigma Chemical Co., St. Louis, MO. Other chemicals and solvents were as indicated previously (12). Ether-extracted porcine pancreatic lipase was obtained from Analabs (North Haven, CT) (200 activity units/mg protein) and a more highly purified preparation was from Sigma Chemical Co. (30,000 activity units/mg protein).

Digestion with pancreatic lipase

The hydrolysis with pancreatic lipase was performed as described by Luddy et al. (14) in the presence of minimal (0.0025%) and physiological (0.35%) taurocholate concentrations. In addition, the substrate/enzyme ratios were altered over a range of 15. The triacylglycerols or ethyl esters (15–25 mg) were dissolved in 100 μl acetone and added to 2 ml digestion buffer made up of 1 M tris[hydroxymethyl]aminomethane (adjusted to pH 8) followed by 0.2 ml 22% $CaCl_2$ solution and 0.5 ml of 0.1% bile salt solution containing the lipase (1–4 mg; 1000–4000 units of enzyme). The digestion was completed in a screw-cap vial in a shaking bath at 37°C over various periods of time and ratios of substrates to give 5 to 95% hydrolysis of the esters. At the end of the digestion the contents of the vial were acidified to pH 1–2 with dilute HCl.

Isolation of lipid classes

The total lipids were extracted by chloroform–methanol 2:1 (12). The lipid classes were separated by normal phase thin-layer chromatography (TLC) using plates prepared in the laboratory from silica gel H containing 7% magnesium acetate (15) and using heptane–isopropyl ether–acetic acid 60:40:3 as developing solvent (12) or from silica gel G containing 5% boric acid and using chloroform–acetone 97:3 as the developing solvent (16).

Preparation of derivatives

Trimethylsilyl (TMS) ethers and esters were prepared as described (13). Diacylglycerols for structural analyses of the original and residual triacylglycerols were obtained by Grignard degradation (17). The *sn*-1,2(2,3)- and 1,3-diacylglycerol products were separated by borate TLC and were converted to the TMS ethers without isomerization. The triacylglycerol fractions were hydrogenated following dissolution in 2 ml methanol to which 1 mg platinum oxide was added prior to exposure to hydrogen gas (18).

Capillary gas–liquid chromatography (GLC)

Total lipid profiles of the organic solvent extracts of the original oil, the alkyl esters, and the extracts of the various digestion and derivatization mixtures were obtained using nonpolar capillary columns containing permanently bonded SE-54 liquid phase (Hewlett-Packard, Palo Alto, CA) as previously described (12). Fatty acid methyl and ethyl esters were resolved according to carbon and double bond numbers using a polar capillary column (15 m × 0.32 mm ID) wall-coated with a cross-bonded film of RTx-2330 (Restek Corp., Port Matilda, PA) as described (19).

RESULTS

Relative rates of hydrolysis of alkyl and glyceryl esters

These were determined by comparing the relative extent of hydrolysis of ethyl and methyl oleate and trioleoylglycerol under conditions where all three substrates were hydrolyzed to a detectable degree. On the basis of the substrate disappearance, it was estimated that from a 1:2:2 (mol/mol/mol) mixture of trioleoylglycerol–methyl oleate–ethyl oleate, the trioleoylglycerol was hydrolyzed 12–15 times faster than the ethyl ester and 2.2–2.8 times faster than the methyl ester at physiological bile salt concentrations (**Table 1**). This difference remained at the somewhat higher rates of hydrolysis obtained in the presence of lower concentrations of bile salts or higher enzyme/substrate ratios. Thus, a simultaneous digestion of equimolar amounts of trioleoylglycerol and methyl and ethyl oleate showed that trioleoylglycerol was hydrolyzed 4–7 times faster than methyl oleate, and methyl oleate was

TABLE 1. Relative rates of hydrolysis of mixed methyl and ethyl oleate and trioleoylglycerol by pancreatic lipase in the presence of a high bile salt concentration

Time of Digestion	Composition of Hydrolysate						Reaction Rate Ratio		
	ME	EE	FFA	MG	DG	TG	TG/ME	ME/EE	TG/EE
min	*mg*						*mole/mole*		
0	4.9	5.3				7.4			
15	4.7	5.2	0.8	0.2	0.7	5.7	2.8	5.4	15.4
30	4.4	5.2	1.6	0.4	0.9	4.5	2.2	5.4	12.1

Assays were performed in the presence of 0.35% sodium taurocholate, 1 mg enzyme protein, and a TG/ME/EE molar ratio of 1/2/2. The values are averages of two determinations. ME, methyl oleate; EE, ethyl oleate; FFA, free oleic acid; MG, monooleoylglycerol; DG, dioleoylglycerol; TG, trioleoylglycerol.

hydrolyzed 5 times faster than ethyl oleate. With increasing proportions of trioleoylglycerol in the digestion mixture, its rate of hydrolysis increased exponentially (**Fig. 1A**), while the relative rates of hydrolysis the alkyl esters decreased (Fig. 1B). With both substrates increasing proportionally, the ratio of the hydrolysis rates remained constant.

Digestion of alkyl esters

Table 2 compares the composition of the residual ethyl esters and the free fatty acids released by pancreatic lipase after 10 to 70% hydrolysis in the presence of a high bile salt concentration. It is seen that the free acid fraction contains significantly less of the 16:3, 16:4, 18:4, 20:5, 22:5, 22:6, and 20:4, and correspondingly more of the 14:0, 16:1, 18:0, and 18:1 acids than the original ethyl esters, especially at the early times. There was a corresponding enrichment in the 16:3, 16:4, 18:4, 20:4, 20:5, 22:5, and 22:6 acids in the residual ethyl esters along with a decrease in the other acids. The proportion of 16:0 remained more or less constant throughout the digestion period. Somewhat more discrimination was obtained in presence of a low bile salt concentration. None of the fatty acids were completely resistant to hydrolysis by pancreatic lipase. By dividing the percent composition of the released fatty acids by the composition of the original esters, it was possible to obtain the relative order of release of the fatty acids, which remained constant at different times and rates of enzyme digestion. On this basis, the relative ease of hydrolysis of the different fatty acids from the ethyl esters can be arranged as follows: 16:1(n-7) > 16:2(n-4) > 14:0 > 18:1(n-9) > 15:0 > 18:1(n-7) > 18:0 > 16:0 > 20:1(n-9) > 18:2(n-6) > 20:4(n-3) > 18:3(n-3) > 16:4(n-1) >> 22:5(n-3) > 16:3(n-4) >> 18:4(n-3) > 20:4(n-6) > 22:6(n-3) > 20:5(n-3). **Fig. 2** (upper two panels) displays this relationship in a graphical form after normalization to palmitate (rate of hydrolysis = 1).

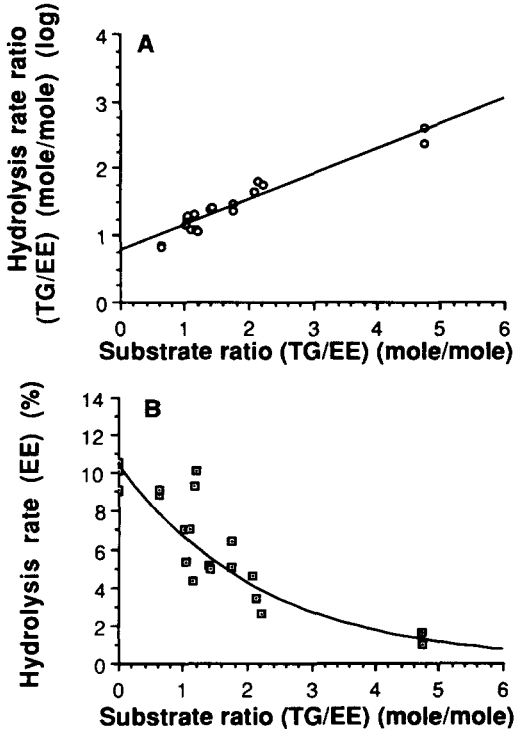

Fig. 1. Correlation between substrate ratio and reaction rate ratio during hydrolysis of mixtures of trioleoylglycerol and ethyl oleate by pancreatic lipase (A) and between substrate ratio and rate of hydrolysis of ethyl oleate (B). Y = 0.764 + 0.382X; r = 0.963; $P<0.005$ (n = 18) and Y = 10.434 × $10^{-0.194x}$, r = 0.853, $P<0.005$ (n = 22), for Fig. 1A and 1B, respectively. TG, trioleoylglycerol; EE, ethyl oleate. Other assay conditions: pancreatic lipase, 2,000 units per assay; sodium taurocholate, 0.0025%; temperature, 37°C; time, 15 min.

Hydrolysis of glyceryl esters

The initial rates of hydrolysis of menhaden oil and corn oil triacylglycerols were compared using equimolar amounts of the substrates. The hydrolysis rates were estimated from the relative total free fatty acid or from the disappearance of the substrates. When expressed as μmol/min per mg enzyme protein, the corn oil and menhaden oils were hydrolyzed at about the same rate (0.30–0.45 μmol/min per mg protein) at the higher bile salt concentration. At the lower bile salt concentration, menhaden oil was hydrolyzed 1.5 times more slowly than corn oil.

High temperature GLC profiles of the reaction mixture indicated that, as the digestion progressed, the residual diacylglycerols and triacylglycerols contained increasingly larger proportions of the higher molecular weight species, while the molecular weight distributions of the free fatty acids and monoacylglycerols approached those of the X-1,3 and *sn*-2 positions of the original triacylglycerols, respectively (results not shown).

Table 3 compares the fatty acid composition of the free fatty acid and monoacylglycerol fractions after 20 and 70% digestion of menhaden oil. The released free fatty acids were low in 16:3, 16:4, 18:4, 20:4, 20:5, 22:5, and 22:6, and high in 14:0, 16:1, and 18:1 species, which correspond to their relative proportions in the *sn*-1 and *sn*-3 positions of the oringinal triacylglycerols. However, a quantitative assessment revealed that much of the 16:3, 16:4, 18:4, 20:4, 20:5, 22:5, and 22:6 acid in the outer positions of the triacylglycerols were resistant to lipase, while 14:0, 16:1, and 18:1 were released in increased proportions. By dividing the percent composition of the released fatty acids by the composition of the *sn*-1 + *sn*-3 positions of the original menhaden oil triacylglycerols, it was possible to construct the order of relative release of the fatty acids, which remained essentially constant at different times and rates of enzyme digestion, e.g., 18:1(n-9) >

TABLE 2. Fatty acid composition of the released free acids and of the residual ethyl esters recovered during hydrolysis of ethyl esters of menhaden oil fatty acids by pancreatic lipase in the presence of a high bile salt concentration

Fatty Acids	Extent of Hydrolysis						
	0%	10%		30%		70%	
	EE	FFA	EE	FFA	EE	FFA	EE
				mole%			
14:0	10.86	14.88	9.73	14.57	9.17	13.53	4.33
15:0	0.67	0.83	0.64	0.81	0.61	0.80	0.37
16:0	18.20	20.70	17.60	20.58	16.88	21.34	11.21
16:1(n-7)	13.79	20.99	11.62	19.90	10.47	17.80	3.35
17:0	0.64	0.63	0.60	0.67	0.62	0.61	0.59
16:2(n-4)	2.35	3.41	2.02	3.33	1.84	3.01	0.72
18:0	2.89	3.71	2.89	3.12	2.82	3.34	2.24
16:3(n-4)	2.11	1.23	2.43	1.15	2.66	1.39	3.79
18:1(n-9)	9.60	12.63	8.51	12.63	7.83	12.23	3.27
18:1(n-7)	3.57	4.03	3.29	4.20	3.10	4.35	1.62
16:4(n-1)	2.34	1.45	2.67	1.64	2.93	1.61	4.10
18:2(n-6)	1.60	1.99	1.44	1.99	1.35	1.97	0.66
18:3(n-3)	1.23	1.40	1.02	1.42	0.97	1.41	0.49
20:1(n-9)	1.53	1.44	1.47	1.57	1.43	1.78	0.93
18:4(n-3)	3.63	1.89	4.21	1.82	4.60	2.15	7.15
20:3(n-6)	0.19	0.23	0.21	0.25	0.21	0.21	0.20
20:4(n-6)	0.89	0.45	0.94	0.44	1.02	0.46	1.82
20:4(n-3)	1.43	1.18	1.48	1.34	1.48	1.50	1.34
20:5(n-3)	13.87	4.33	16.61	4.44	18.45	5.60	33.25
22:5(n-3)	1.86	1.04	2.08	1.21	2.18	1.57	2.72
22:6(n-3)	7.10	2.31	8.52	2.65	9.38	3.57	15.87

Digestion conditions as in Table 1, except there was 4 mg enzyme protein/assay. Values are averages of two determinations for each time point.

16:1(n-7) > 16:2(n-4) > 18:2(n-6) > 18:1(n-7) > 20:1(n-9) > 14:0 > 18:3(n-3) > 15:0 > 20:4(n-3) > 18:0 > 16:0 > > 22:5(n-3) > 20:4(n-6) > > 18:4(n-3) > 16:4(n-1) > 16:3(n-4) > > 20:5(n-3) > 22:6(n-3). These data are displayed in graphical form in Fig. 2 (lower two panels) following normalization to palmitate. The order is closely similar to that determined for the ethyl esters.

A comparison of the fatty acid composition of the released 2-monoacylglycerols with the known composition of the fatty acids in the *sn*-2 position indicated relatively little change. There was only a gradual increase in the proportion of the long chain species, especially of 20:5, 22:5, and 22:6 as hydrolysis time progressed. By dividing the percent composition of the released monoacylglycerols by the composition of the *sn*-2 position of the original menhaden oil triacylglycerols, it is possible to obtain a relative order of release of the 2-monoacylglycerols, which differed markedly from the order of release of the fatty acids, e.g., 18:0 > > 20:4(n-3) > 20:4(n-6) > 20:5(n-3) > 22:6(n-3) > 22:5(n-3) > 16:2(n-4) > 17:0 > 18:1(n-7) > 18:1(n-9) > 20:1(n-9) > 18:3(n-3) > 16:1(n-7) > 16:0 > 16:4(n-1) > 16:3(n-4) > 15:0 > 18:2(n-6).

Table 3 also compares the fatty acid composition of the residual *sn*-1,2(2,3)-diacylglycerols after 20% and 70% digestion of menhaden oil. It is seen that the diacylglycerol fractions contained increased proportions of the long chain polyunsaturated fatty acids. However, the increase in the proportions of the long chain polyunsaturates was much greater than would by anticipated on the basis of the known proportion of these acids in the *sn*-2 position and from the relative release of these acids from the X-1(3) positions, which suggests that a further discrimination occurred against diacylglycerols containing two long chain acids and against triacylglycerols containing two or three long chain fatty acids per molecule (see below).

Fig. 3 compares the polar capillary GLC profiles of molecular species for the *sn*-1,2(2,3)-diacylglycerols derived from residual triacylglycerols by Grignard degradation and the residual *sn*-1,2(2,3)-diacylglycerols isolated following 70% hydrolysis of the menhaden oil. The quantitative values of the major species are given in **Table 4**, along with the values for the *sn*-1,2(2,3)-diacylglycerols derived from the original triacylglycerols. It is seen that the residual acylglycerols, which showed the greatest resistance to pancreatic lipase, represent species containing at least one residue of 16:3(n-4), 16:4(n-1), or 20:5(n-3) in combination with one long chain fatty acid per acylglycerol molecule. In comparison to the polar capillary GLC profiles of the *sn*-1,2(2,3)-diacylglycerols derived by Grignard degradation of the original fish oil,

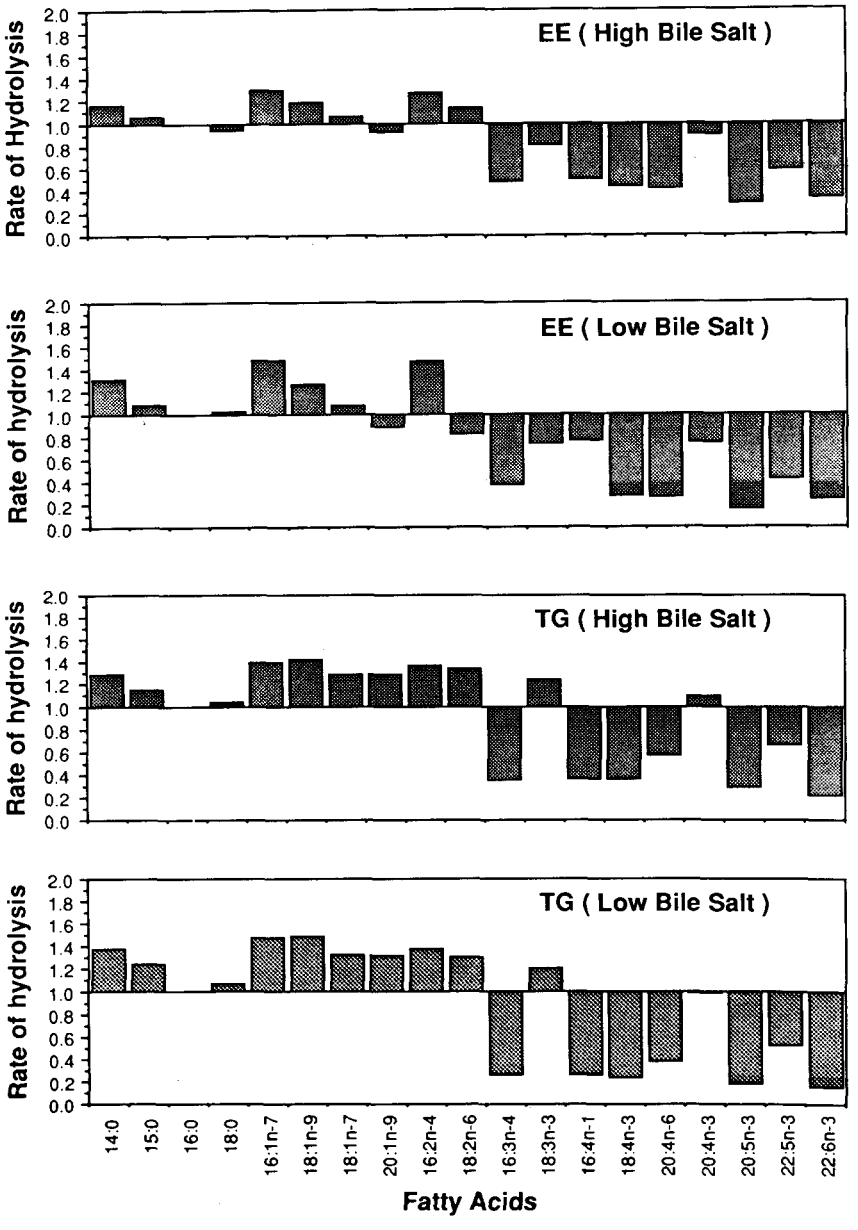

Fig. 2. Relative order of fatty acid release during 30% hydrolysis of menhaden oil triacylglycerols and its ethyl esters by pancreatic lipase. The order of release was determined by dividing the percent composition of the released acids by the percent composition of the acids in the original ethyl esters or in the primary positions of the original triacylglycerols and normalizing to palmitic acid. TG, menhaden oil triacylglycerols; EE, ethyl esters of menhaden oil fatty acids; high bile salt, 0.35% sodium taurocholate; low bile salt, 0.0025% sodium taurocholate. Average of two to three determinations.

there was a dramatic increase in the species containing two polyunsaturated long chain fatty acids per molecule of the residual diacylglycerols, e.g., 16:3-20:5, 18:1-20:5, 16:3-22:6, 18:0- 22:6, 18:0-22:5, 20:5-20:5, 18:4-22:6, 18:4-22:5, and 20:5-22:6.

Table 5 compares the carbon number distribution of menhaden oil triacylglycerols and the residual triacylglycerols after 70% hydrolysis of the oil. For this purpose the triacylglycerols were hydrogenated to improve the resolution and recovery of the longer chain lengths. There was a marked increase in the proportion of the species containing two and three long chain fatty acids (C_{20} and C_{22}) per triacylglycerol molecule in the residual triacylglycerols. There is a clear indication that as the hydrolysis proceeds, there is an increase in the proportion of triacylglycerols containing two or three long chain fatty acids per molecule. This indicates that the rate of release of a long chain fatty acid by pancreatic lipase may be further decreased by the presence one or two other long chain acids in the same triacylglycerol molecule.

TABLE 3. Fatty acid composition of the free acid, monoacylglycerol, and diacylglycerol fractions before and after 20 and 70% hydrolysis of menhaden oil by pancreatic lipase in the presence of a high bile salt concentration

Fatty Acids	Hydrolysis Products								
	Free Fatty Acids			Monoacylglycerols			Diacylglycerols[c]		
	0%[a]	20%	70%	0%[b]	20%	70%	0%	20%	70%
	mole%								
14:0	8.86	12.52	11.32	14.38	12.19	11.93	10.50	10.76	8.65
15:0	0.60	0.76	0.68	0.93	0.76	0.70	0.68	0.68	0.52
16:0	18.85	19.53	19.00	22.81	21.80	19.67	19.55	19.18	16.63
16:1(n-7)	12.94	19.65	18.46	14.51	14.01	11.93	13.52	12.05	9.59
17:0	1.03	0.68			0.35		0.92	0.56	0.82
16:2(n-4)	2.13	3.15	3.03	2.55	2.78	2.25	2.26	2.20	1.76
18:0	4.02	4.20	4.10	1.04	1.08	0.53	2.54	2.23	1.83
16:3(n-4)	1.99	0.74	0.95	3.07	2.77	3.26	2.19	2.66	3.10
18:1(n-9)	11.07	16.44	16.47	4.12	4.11	3.26	8.19	7.03	4.81
18:1(n-7)	3.97	5.23	5.18	1.43	1.44	1.14	2.94	2.60	1.96
16:4(n-1)	1.94	0.81	1.08	3.70	3.48	3.87	2.42	2.52	3.28
18:2(n-6)	1.63	2.29	2.28	1.24	1.01	1.07	1.47	1.05	1.35
18:3(n-3)	1.28	1.67	1.62	0.95	0.93	0.78	1.14	1.00	0.74
20:1(n-9)	1.68	2.14	2.38	0.52	0.51	0.36	1.27	1.24	0.88
18:4(n-3)	3.56	1.36	1.66	3.69	4.41	4.34	3.67	4.18	5.34
20:3(n-6)	0.21	0.26	0.21	0.10	0.18	0.09	0.15	0.19	0.11
20:4(n-6)	0.73	0.57	0.37	0.68	0.78	0.98	0.79	0.88	1.36
20:4(n-3)	1.48	1.59	1.91	0.59	0.69	0.65	1.30	1.27	1.08
20:5(n-3)	13.24	4.11	5.25	11.74	13.11	15.12	13.72	16.36	24.01
22:5(n-3)	1.20	0.82	1.08	2.80	2.95	3.47	2.38	2.38	2.48
22:6(n-3)	6.62	1.66	2.23	9.78	10.80	13.49	8.60	8.98	9.97

Values are averages of two to three determinations.
[a]The X-1(3)-positions of menhaden oil triacylglycerols.
[b]The 2-position of menhaden oil triacylglycerols.
[c]Residual *sn*-1,2(2,3)-diacylglycerols.

DISCUSSION

Assay conditions

The digestion conditions were modeled on those described by Luddy et al. (14), which had been successfully utilized by Bottino, Vandenburg, and Reiser (3) in the studies on whale and menhaden oil triacylglycerols. The enzyme/substrate ratios were optimized to provide readily measurable rates of hydrolysis of all primary esters of fatty acids in the mixture. In some instances the reactions were performed under conditions where the enzyme was saturated with the substrate, while in others the enzyme/substrate ratio varied. Increasing the concentration of bile salts to physiological levels resulted in partial inhibition of the reaction rate as already noted by Mattson and Volpenhein (20). This is apparently due to a displacement of the lipase from the emulsion surface in the absence of sufficient co-lipase (21). However, addition of more enzyme always resulted in increased hydrolysis. It is possible that pancreatic carboxylic ester hydrolase, if present in the crude enzyme preparation, could have contributed a maximum of 0.5% of the rate of pancreatic lipase-colipase system (22) in the presence of bile salt. Without bile salt the esterase is not active with insoluble substrates. There was no evidence for inhibition of the lipase activity by the released free fatty acids (4) or for a resynthesis of glyceryl or alkyl esters by the lipase under our working conditions. In the presence of an excess triacylglycerol, the rate of hydrolysis of the alkyl esters was decreased. This suggests that the methyl and ethyl esters were unable to compete effectively with the triacylglycerols for occupancy of the emulsion surface where hydrolysis is believed to take place. This may have been due to the lower polarity of the ethyl esters compared to triacylglycerols and their greater solubility in the triacylglycerol interior of the lipid particles. There were no signs of deterioration of the enzyme with prolonged incubation time (up to 8 h). For the purposes of discussion it was assumed that porcine pancreatic lipase possesses the same fatty acid and ester specificity as rat pancreatic lipase.

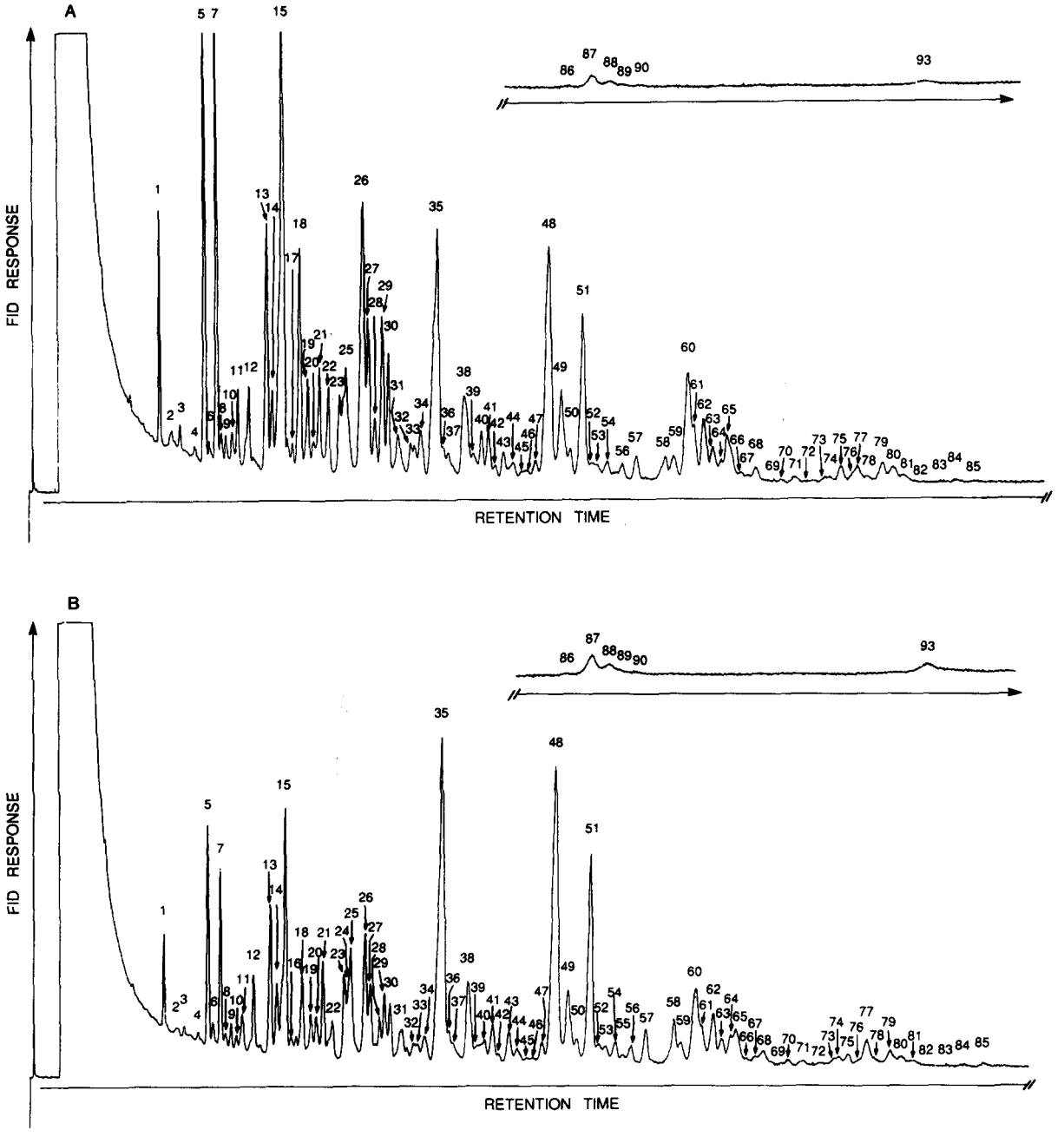

Fig. 3. Comparison of polar capillary GLC profiles of molecular species of (A) the *sn*-1,2(2,3)-diacylglycerols derived from residual menhaden oil triacylglycerols by Grignard degradation and (B) residual *sn*-1,2(2,3)-diacylglycerols isolated after 70% hydrolysis of menhaden oil triacylglycerols with pancreatic lipase. Peak identification is as in Table 4. GLC conditions: instrument, Hewlett-Packard capillary gas chromatograph Model 5880 equipped with a polar capillary column (15 m × 0.32 mm ID) wall-coated with cross-bonded film of RTx-2330 (Restek Corp., Port Matilda, PA). Carrier gas, H_2 at 3 psi head pressure; temperature program, 240–260C at 1°C/min, then isothermal at 260°C. Sample, 1 μl of approx. 0.1% solution of TMS-treated lipid mixture in hexane.

Resistance of fish oil triacylglycerols

The in vitro hydrolysis of menhaden oil has been previously investigated by Bottino et al. (3), who concluded that the polyunsaturated fatty acids with double bonds close to the carboxyl group are resistant to attack by pancreatic lipase. This resistance was rationalized as a result of shielding by the terminal methyl group. Brockerhoff (4) later demonstrated that unsaturated fatty acids with double bonds at C_2 to C_5 are resistant to hydrolysis. Later Heimermann et al. (23) reported that isomeric *cis*-octadecadienoic acids with double bonds in positions Δ^2 to Δ^7 were discriminated against during triacylglycerol

TABLE 4. Composition of molecular species of *sn*-1,2(2,3)-diacylglycerol moieties of original triacylglycerols of menhaden oil and of residual triacylglycerols and diacylglycerols after 70% hydrolysis with pancreatic lipase

Peak No.	Molecular Species	Triacylglycerols		Residual Diacylglycerols
		Original	Residual	
			mole%	
1	14:0-14:0	1.62	1.20	0.75
5	14:0-16:0	3.36	3.00	2.10
7	14:0-16:1(n-7)	3.80	3.07	1.74
11	14:0-16:2(n-4)	0.66	0.62	0.53
12	15:0-16:1 + 14:0-16:3(n-4)	0.84	0.84	0.94
13	16:0-16:0	3.41	2.56	2.35
14	14:0-16:4(n-1)		0.68	0.96
15	14:0-18:1(n-9) + 16:0-16:1(n-7) + 14:0-18:1(n-7)	8.12	6.83	4.35
18	16:1(n-7)-16:1(n-7)	2.61	2.17	1.28
19	16:0-16:2(n-4)	1.27	1.01	0.72
21	16:0-16:3(n-4)	1.43	1.17	1.30
22	16:1(n-7)-16:2(n-4)	0.98	0.88	0.70
23	16:1(n-7)-16:3(n-4)	0.84	0.74	1.65
25	16:0-16:4(n-1) + 16:0-18:0	2.18	1.98	1.32
26	16:0-18:1(n-9)	4.73	4.80	2.41
27	16:0-18:1(n-7) + 16:1(n-7)-18:0	2.16	1.69	1.22
28	16:1(n-7)-16:4(n-1) (late sh of pk 30)	0.00	0.66	0.66
29	16:1(n-7)-18:1(n-9)	1.90	1.90	1.07
30	16:1(n-7)-18:1(n-7) + 16:0-18:2(n-6)	1.27	1.38	0.94
32	16:1(n-7)-18:2(n-6)	0.45	0.45	0.21
33	18:1(n-9)-16:2(n-4)	0.47	0.37	0.21
34	16:0-18:3(n-3)	0.69	0.77	0.54
35	16:0-18:4(n-3) + 18:1(n-9)-16:3(n-4) + 14:0-20:5(n-3)	5.24	6.17	9.47
38	16:1(n-7)-18:4(n-3) + 18:1(n-9)-16:4(n-1) + 18:0-18:1	2.49	2.07	1.95
40	18:1(n-9)-18:1(n-9)	0.91	0.94	0.32
41	18:1(n-9)-18:1(n-7) + 14:0-21:5(n-3) + 15:0-20:5(n-3)	0.68	0.97	0.74
48	16:0-20:5(n-3)	5.19	8.04	9.92
49	14:0-22:5(n-3) + 14:0-22:6(n-3)	2.18	2.87	2.19
50	16:1(n-7)-20:3(n-3) + 18:1(n-9)-18:3(n-3)	0.59	0.59	0.53
51	16:1(n-7)-20:5(n-3)	3.10	3.97	5.53
54	16:0-21:5 + 17:0-20:5		0.54	0.61
58	16:3(n-4)-20:5(n-3)	0.61	0.70	1.33
59	18:0-20:5(n-3)	1.10	0.66	0.54
60	16:0-22:6(n-3)	5.42	6.32	6.26
62	18:1(n-9)-20:5(n-3)	2.16	2.11	2.55
63	18:1(n-7)-20:5(n-3)	1.52	1.20	1.31
65	16:1(n-7)-22:6(n-3)	2.15	2.37	2.09
73	16:3-22:6		0.22	0.84
75	16:4(n-1)-22:6(n-3) + 16:4(n-1)-22:5(n-3)	0.72	0.78	0.68
76	18:0-22:6(n-3) + 18:0-22:5(n-3)	1.49	0.97	2.39
79	18:1(n-9)-22:6(n-3) + 18:1(n-9)-22:5(n-3)	1.70	1.13	1.17
80	18:1(n-7)-22:6(n-3) + 18:1(n-7)-22:5(n-3)	1.31	0.87	0.67
87	20:5(n-3)-20:5(n-3)	0.85	1.01	1.75
88	18:4-22:6 + 18:4-22:5	0.60	0.48	1.01
93	20:5-22:6 (C_{42})	3.90	2.96	4.87
Other		14.22	13.76	13.66

Fatty acid identification according to Ackman (30).

hydrolysis by pancreatic lipase in vitro. The present study shows that the relative rates of hydrolysis of the different fatty acids depend on the location of the double bonds (Δ^4-Δ^7), but not the chain length (C_{16}-C_{22}) and degree of unsaturation (1-6 double bonds). Furthermore, there was evidence that menhaden oil triacylglycerols containing two or three long chain polyunsaturated fatty acids per molecule were more resistant to pancreatic lipolysis than those with one polyunsaturated fatty acid. However, none of the fatty acid esters of glycerol or ethanol was completely resistant to hydrolysis by pancreatic lipase. In fact, the rate of release of the most resistant acid, 22:6(n-3), differed from that of the least resistant fatty acid, 18:1(n-9), only by a factor of 6. It was therefore felt that at rapid rates and extensive hydrolysis, the lumenal free fatty acid composition would approximate that of the original ethyl esters or of the outer positions of the dietary triacylglycerol as observed in our recent study (12). It has

TABLE 5. Carbon number distribution of residual triacylglycerols after 20 and 70% hydrolysis of menhaden oil by pancreatic lipase and of original menhaden oil triacylglycerols

Carbon Numbers	Residual Triacylglycerols		Original Triacylglycerols
	20%	70%	
		mole%	
42	0.07		
43			
44	0.81	0.22	1.62
45		0.08	0.65
46	3.41	1.65	5.41
47	0.59	0.42	1.55
48	7.93	4.98	10.94
49	1.36	0.97	2.05
50	12.63	10.42	15.60
51	1.88	1.65	1.67
52	16.02	16.72	17.90
53	2.18	2.13	2.23
54	14.81	17.85	14.91
55	2.00	1.88	1.50
56	11.77	16.02	10.52
57	1.67	1.31	0.87
58	8.02	11.28	6.09
59	1.43	0.95	0.48
60	5.09	6.72	3.05
61	1.38	0.47	0.31
62	3.27	2.91	1.24
63	1.39	0.16	0.29
64	2.29	0.83	0.40
65		0.04	
66	1.20	0.27	

Values are averages of two to three determinations.

been estimated (24) that under normal physiological conditions the lumenal concentration of pancreatic lipase in humans may exceed the concentration necessary for complete hydrolysis of dietary fat by a factor of 1,000. There was little difference between menhaden and corn oil in the initial rates of hydrolysis. This appears reasonable since both oils contain high proportions of triacylglycerols, which do not contain polyunsaturated fatty acids in the primary positions. Such triacylglycerols would be attacked first and at comparable rates.

The release of the 2-monoacylglycerols from menhaden oil has not been previously investigated. The present study shows that the 2-monoacylglycerol composition changes very little during the course of the in vitro lipolysis. This is in agreement with the results of the lumenal hydrolysis of this oil (12). The observation can be explained on the basis of a noncorrelative association of the fatty acids in natural triacylglycerols (17). In such a case the presence of every fatty acid in each position is completely independent of the concentration or positional location of any other fatty acid. Hence it would be anticipated that the 2-monoacylglycerols released by removal of any of the fatty acids in the outer positions would be identical, as observed experimentally. This is in general agreement with the analyses of the molecular species of the *sn*-1,2(2,3)-diacylglycerol intermediates of the digestion and with the *sn*-1,2(2,3)-diacylglycerols generated by Grignard degradation from the residual menhaden oil triacylglycerols. The accumulation of the long chain diacylglycerols is in the proportion anticipated from the slower release of the polyunsaturated fatty acids from the primary positions and the proportion of long chain acids in the *sn*-2 position. The accumulation of the longer chain fatty acids in the 2-monoacylglycerols at later times may be attributed to slower isomerization to the primary positions, from which they may be released by the lipase.

Resistance of alkyl esters

Triacylglycerols are the most active substrates for pancreatic lipase, but alkyl esters of fatty acids are also attacked. Sarda and Desnuelle (25) have reported that methyl oleate is hydrolyzed at the rate 1/30 that of trioleoylglycerol. Brockerhoff (26) has discussed the behavior of various alkyl esters of oleic acid in comparison to trioleoylglycerol and concluded that lipolysis is promoted by electrophilic substituents. Hence the methyl, and more so the ethyl, esters would be expected to be hydrolyzed more slowly than the corresponding triacylglycerols, although the differences in rates cannot be quantitatively predicted. The present data indicate that at equimolar substrate ratios, trioleoylglycerol is hydrolyzed 5 times faster than methyl oleate, which is hydrolyzed 5 times faster than ethyl oleate. Our relative rates of hydrolysis of the methyl and ethyl esters of fatty acids are comparable to those of Savary (27). The somewhat higher rate of hydrolysis of the ethyl esters of the shorter chain (C_{12} and C_{14}) when compared to the longer chain (C_{16} and C_{18}) saturated fatty acids was also in agreement with the observations of Savary (27). This effect cannot be readily explained in terms of enzyme specificity or orientation of the ester molecules at the oil–water interface and may involve differences in substrate emulsification. In an earlier study by Mattson and Volpenhein (28), the common C_4–$C_{18:1}$ esters of ethanol were found to be hydrolyzed at nearly identical rates, indicating complete absence of chain length effects.

In general, the menhaden oil fatty acids were released from the ethyl esters in essentially the same order as those from the primary positions of the triacylglycerols of menhaden oil. There were a few changes in the order of release of the minor components, which may be attributed to the fact that the composition of the minor components differed between the ethyl esters and the primary positions of the triacylglycerols. In both instances, the acids with Δ^4–Δ^7 double bonds were released more slowly than the other fatty acids. This shows that the resistance to lipolysis is not confined to the glyceryl esters of long

chain polyunsaturated fatty acids. Likewise, the ethyl esters also showed the same chain length specificity for the saturated and monounsaturated acids as the glyceryl esters.

Significance of findings

Since the relative rates of release of the various fatty acids differ by a factor of 6 or less, measurable differences in composition between the released and original fatty acids can be seen readily only at low rates of hydrolysis. At high rates of hydrolysis brought about by a high enzyme to substrate ratio, all the fatty acids are released within a short period of time including those with Δ^4-Δ^7 double bonds. Therefore, given sufficient time and sufficient excess of enzyme, little difference would be expected between the released and bound fatty acids. It is suggested that such conditions exist in the intestinal lumen, where representative release of fatty acids has been observed from both menhaden oil and from the corresponding fatty acid ethyl esters (12). This hypothesis is supported by the reported 1,000-fold excess of pancreatic lipase activity in the lumen (24). We have previously shown (12) that the micellar solution remains saturated with free fatty acids and monoacylglycerols during the digestion and absorption of menhaden oil.

The 10- to 50-fold lower rate of hydrolysis of the ethyl esters, however, may be more difficult to overcome by increased rates of hydrolysis due to a high enzyme substrate ratio. Nevertheless, the lumenal rates of hydrolysis of the ethyl esters of menhaden oil fatty acids obtained in our previous experiments (12) also appeared to maintain saturated micellar solutions in the lumen during the digestion and absorption of the ethyl esters, which suggests that the rate of hydrolysis of the ethyl esters also exceeded the rate of absorption of the free fatty acids. It is possible that the lower lymphatic and plasma recoveries of fatty acids from ethyl ester feeding (6, 7) are due to the necessity, in the absence of 2-monoacylglycerols, to resynthesize the triacylglycerols via the less efficient phosphatidic acid pathway (29).

When menhaden oil or the corresponding fatty acid ethyl esters are taken as dietary supplements, the digestion and absorption of the fish oil fatty acids may take place in the presence of fatty acids and monoacylglycerols originating from other oils and fats, and the relative rates of digestion of the glyceryl and ethyl esters may vary with the exact nature of the other dietary fat. The present study shows that the relative rates of ethyl ester hydrolysis are decreased in the presence of an excess of triacylglycerols. The actual rates depend on the specific ratio of the two ester types. The ethyl esters were hydrolyzed most readily in the absence of triacylglycerols, but the released fatty acids would by expected to be absorbed most readily in the presence of 2-monoacylglycerols. The role of the phosphatidic acid pathway in the mucosal resynthesis of dietary triacylglycerols and in maintaining low lymphatic triacylglycerol recoveries requires further investigation.

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