Lymphatic Absorption of Nonvolatile Oxidation Products of Heated Oils in the Rat

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ABSTRACT

The lymphatic absorption of nonvolatile oxidation products (NVOP) formed during heating of fats was studied. Heated colza or soybean oils or synthetic triglycerides containing a definite aromatic or alicyclic fatty acid were fed to thoracic duct-cannulated rats. Tritium-labeled triolein was added to each dietary fat, as an internal standard, in order to calculate the percentage of lymphatic absorption of the ingested NVOP. Results show that 4% of the total polymeric acids, 53% of the total oxidized monomeric acids and 96% of the total cyclic monomeric acids were recovered in the lymphatic lipids. Gas liquid and quantitative thin layer chromatography of these 3 classes indicated that, within a NVOP class, the various constituents did not present the same absorption rate. The lymphatic absorptions of individual oxidized monomers were between 25 and 93%. Concerning the polymer fraction, the lymphatic recoveries were 1% (nonpolar dimers), 6.8% (polar dimers) and 12% (polar oligomers). Aromatic acids were absorbed to a lesser degree (50-60%) than cyclohexenic acids (91-98%).

INTRODUCTION

The heating of oils under the conditions used for domestic frying leads to the formation of volatile breakdown derivatives and nonvolatile oxidation products (1-8). Nonvolatile oxidation products (NVOP) have triggered numerous nutritional studies. Because they accumulate in the heated oil, they are subsequently ingested with the fried foods. Furthermore, their analysis has shown the presence of potentially toxic compounds (5). However, though the toxic effects are dependent on their intestinal absorption, their uptake into the lymph has only been determined in a limited number of cases (9-12). In these instances, they were generally fed to the animals in the form of methyl esters, whereas in heated oils they are essentially present as glycerides (13). Insofar as results obtained with natural fatty acids (14) can be extrapolated to their thermal oxidation derivatives, the possibility that the lymphatic recovery of NVOP is lower when they are ingested as methyl esters rather than as glycerides cannot be excluded. As a matter of fact, the natural fatty acid methyl esters are not absorbed intact by the intestinal mucosa, even when they are fed with triglycerides to favor their micellar solubilization in the intestinal lumen (14). On the other hand, the hydrolysis of normal fatty acid methyl esters by pancreatic lipase is considerably slower than that of the corresponding triglycerides (15-17). In NVOP, the hydrolysis rate of methyl esters probably is even lower. Indeed, heated oil itself is not as rapidly hydrolyzed as the corresponding fresh oil (18).

In this study, thoracic duct-cannulated rats were fed either heated oils of a known nonvolatile oxidative product (NVOP) composition or triglycerides containing a synthetic model NVOP. The lymphatic recovery of ingested NVOP was estimated. However, an exact determination of the absorption rate must take into account the dilution of lymphatic lipids of dietary origin, such as NVOP, by those of endogenous origin (19-21). Consequently, a radioactive internal standard (glycerol tri[9,10-^{3}H] oleate), of which the intestinal absorption is complete (22), was added to each studied dietary fat. The endogenous dilution was calculated by comparison of the specific radioactivities of the total fatty acids from dietary and lymphatic lipids, respectively.

MATERIALS AND METHODS

Dietary Fats

Heated oils. Refined soybean oil was heated 14 times for 30 min at 220 C in a stainless steel commercial-type deep fat fryer. Between the different heating periods, which were separated by a one-day interval, the oil was allowed to cool to room temperature in the same receptacle. A Primor colza oil was continuously heated for 12 hr at 275 C in a nitrogen atmosphere in order to obtain a higher content in cyclic monomeric derivatives than is usually formed in household fryings. These heated oils were fed to the animals without any previous fractionation.

Synthetic triglycerides containing a cyclic monomeric acid. All syntheses were done by Dr. J. Graille and Mrs. P. Perfetti. 9-(2′-Propyl benzene) nonanoic acid was synthesized accord-

ing to Friedrich (23). Its purity (> 98%) was verified by gas liquid chromatography (GLC) and its structure confirmed by infrared (IR) spectroscopy and nuclear magnetic resonance (NMR). A mixture containing 10% of this acid and 90% of pure olive oil total fatty acids was used to esterify the glycerol directly at 130 C, under nitrogen and in the presence of *p*-toluene sulfonic acid (0.2% by weight of the fatty acids). The triglyceride obtained in this way was then purified by chromatography on a Florisil column. The GLC analysis showed that the aromatic acid was unmodified during esterification and represented 9.5% of the triglyceride total fatty acids.

9-(2′-Propyl cyclohex-4′-en-1′-yl) nonanoic acid was synthesized as previously described (24). Analysis demonstrated the presence of a mixture containing the expected cyclohex-4′-en isomer (70%) and 2 positional isomers (20 and 10%) (24). Pure olive oil total fatty acids were added to this isomer mixture. The corresponding acyl chlorides were prepared and condensed with glycerol in the presence of pyridine (25). The triglyceride was finally purified on a Florisil column.

Procedures for Lymph Cannulation and Lipid Extraction

A permanent thoracic duct cannulation was performed, as indicated by Bollman et al. (26) on male Wistar rats weighing 200-250 g. The rats were placed in restraining cages and allowed free access to a 0.9% NaCl solution and a fat-free diet of boiled rice. A few hours after the surgical operation, when a regular flow of lymph was achieved, the fat (0.7 ml of test fat + 0.53 nmol of [^{3}H]triolein, specific radioactivity = 0.414 μCi/nmol) followed by 1 ml of 0.9% NaCl solution were given to each rat by stomach tube under slight ether anesthesia. The animals were then replaced in their restraining cages, with free access to the saline solution and boiled rice. During the 48 hr following the fatty meal, the lymph was collected without fractionation in a tube maintained in an ice bath. At least 7 cannulated rats were used for each studied fat. The corresponding lymphs were combined and the lipids directly extracted using the Folch et al. (27) method. No measurable NVOP quantities were found in the discarded aqueous solutions.

Total NVOP Assay

The procedure described by Naudet and Biasini (28) was essentially followed except that, after the saponification step, an ether extraction according to the IUPAC method (29) was preferred. This was, in fact, the only method which allowed the total elimination of unsaponifiable material from the lymphatic lipids without any NVOP loss, as verified by chromatography. The total fatty acids (normal and NVOP) were separated by thin layer chromatography (TLC) on Silica Gel G (Merck) and the NVOP were directly determined on the plate, by photodensitometry after charring of compounds (28). The total NVOP were conventionally expressed in ricinoleic acid weight, by reference to a standard curve (28).

Cyclic Monomeric Acid Assay

Total fatty acids, free from unsaponifiable material, were prepared as previously described and then methylated with 4% anhydrous HCl in methanol (30). In the heated oils and corresponding lymphatic lipids, the fraction containing all of the normal and cyclic monomeric esters was isolated from the total methyl esters by alumina column chromatography (31). This fraction was then hydrogenated before GLC analysis (32). In this way, the cyclic derivatives were eluted between methyl stearate and methyl arachidate, without any overlapping with the normal fatty acid methyl esters (32). In the experiments with the 2 model cyclic acids, the total methyl esters were directly analyzed by GLC. Fractionation on alumina and hydrogenation are unnecessary in this case because of the GLC retention times of the model compounds (see Results).

The methyl esters were separated on a 10 ft. x 1/8 in. stainless steel column packed with 7% butanediol succinate (BDS) on gas chrom Q (100-120 mesh). The carrier gas (nitrogen) flow was maintained at 30 ml/min and the column temperature at 185 C. The chromatograms were quantified using a Varian model CDS 111 integrator.

Polymeric and Oxidized Acids Assay

All the methods for these assays have been published. The dietary or lymphatic triglycerides were completely hydrolyzed by the nonspecific *Candida cylindracea* lipase (33). After removal of the unsaponifiable material (29), the acids were isolated and methylated using methyl sulfate at room temperature. These conditions do not modify the NVOP structure (33). The methyl esters were then separated into 2 fractions by a reversed-phase partition chromatography on a polyethylene powder column (33). The first fraction contained esters of the oxidized monomeric acids which were quantified after TLC, charring and photodensitometry (28,34). The second fraction eluted

from the polyethylene column was subjected to TLC (30) in order to separate the polymeric acid esters from the other constituents (esters of cyclic monomeric acids and of normal fatty acids). The polymers were determined, as before, by photodensitometry after charring.

Radioactivity Measurements

Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer, using a POPOP (100 mg/ℓ)-PPO (3 g/ℓ) toluene solution as scintillation mixture.

Calculation of Recovered NVOP

The lymphatic recovery of NVOP was calculated as follows. First, from the specific radioactivities of the total fatty acids respectively isolated from the dietary (χ, CPM/g) and lymphatic (x, CPM/g) lipids, an "isotopic dilution factor" ($f = \frac{\chi}{x}$) was calculated. Because of the dilution of lymphatic lipids of dietary origin by endogenous lipids (19-21), this factor is higher than one. Secondly, from the quantitative analysis of the 2 already mentioned fatty acid fractions, the weight percentage of each studied NVOP present in the dietary (Y) and lymphatic (y) total fatty acids was calculated.

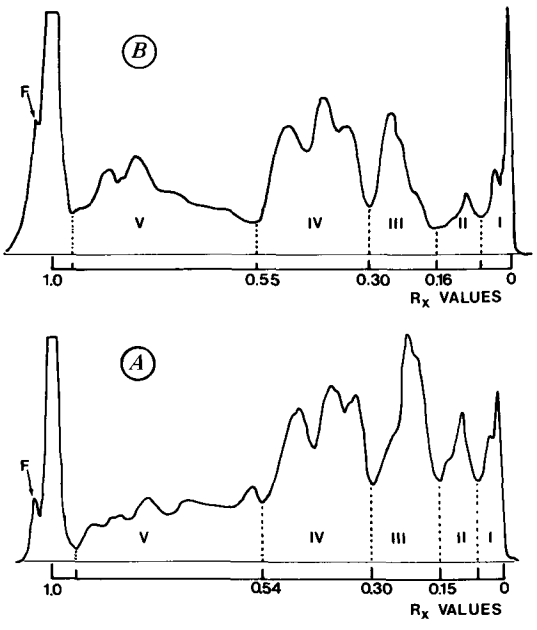

FIG. 1. Photodensitograms of the oxidized monomeric acid fraction in heated soybean oil (A) and in lymphatic lipids (B). The oxidized acids were separated by TLC as methyl esters (see Materials and Methods). R_X = distance of the oxidized ester from the starting point/distance of methyl stearate from the starting point. F - solvent front.

The term $\frac{y}{Y} \cdot 100$ corresponds to the apparent lymphatic absorption which does not take into account the endogenous dilution. The true percentage of ingested NVOP recovered in the lymph (R) was given by $R = f \cdot \frac{y}{Y} \cdot 100$.

RESULTS AND DISCUSSION

In a first experiment, 9 thoracic duct-cannulated rats were given the heated soybean oil containing tritium-labeled triolein. The lymph was collected for 48 hr in order to take into account the delay in absorption of the NVOP (10). During this short test-period, the rats never suffered diarrhea. The total fatty acids isolated from the dietary and lymphatic lipids had specific radioactivities equal to 415,000 CPM/g and 300,000 CPM/g, respectively. From these values, an isotopic dilution factor equal to 1.38 was calculated. Moreover, these 2 fatty acid fractions contained 9.8% and 2.9% total NVOP, respectively. These data were used to calculate that 41% of the total ingested NVOP were absorbed through the lymph. A value of 31% has been obtained for the same lymph collection time (10). However, in the 31% results, the total ^{14}C-labeled methyl esters from heated oil, and not the oil itself, were fed to the rats. The difference between these two results suggests that the intestinal absorption of NVOP is better when they are ingested as glycerides rather than as methyl esters. From this point of view, NVOP would behave like natural fatty acids (14).

Intestinal Absorption of Oxidized Monomeric Acids

The oxidized monomer fraction, isolated by polyethylene column chromatography, represents 4.7 and 1.8%, respectively, of the total fatty acids in the heated soybean oil and the corresponding lymphatic lipids. Taking into account the previously determined isotopic dilution factor, 1.38, it can be calculated that 53% of the oxidized monomeric dietary derivatives, considered as a whole, were recovered in the lymph within 48 hr after administration of the fat.

Given the structural diversity of the oxidized compounds likely to be present in a heated oil (5), a comparison of the composition of the oxidized acid fraction in the dietary and lymphatic lipids seemed interesting in order to see if all the constituents were equally absorbed through the lymph. Such a detailed study of this NVOP class does not seem, as yet, to have been done. Typical photodensitograms obtained after TLC of these 2 fractions are shown in Figure 1. On the whole, the 2 photodensito-

TABLE I

Relative Composition of the Dietary and Lymphatic Monomeric Oxidized Acids and Lymphatic Recovery

	Total fatty acids (%)		Total monomeric oxidized acids (%)		
	Heated soybean oil	Lymphatic lipids	Heated soybean oil	Lymphatic lipids	Lymphatic absorption[a] (%)
Total oxidized acids	4.70	1.80	100	100	53
Fraction 1[b]	0.24[c]	0.16[c]	5.0	8.7	93
Fraction 2	0.50[c]	0.09[c]	10.7	5.1	25
Fraction 3	1.15[c]	0.30[c]	23.9	16.3	36
Fraction 4	1.71[c]	0.68[c]	36.5	38.1	55
Fraction 5	1.12[c]	0.57[c]	23.9	31.8	70

[a]The isotopic dilution factor 1.38 was used to calculate these values (see Materials and Methods).
[b]The numbers correspond to the fraction numbers indicated on the photodensitograms in Fig. 1.
[c]Calculated compositions. The other compositions were obtained from chromatographic data.

grams are qualitatively similar, suggesting that the various oxidized monomers from heated soybean oil are recovered in the lymphatic lipids. However, the multiplicity of the compounds, added to their incomplete TLC separation, did not allow an accurate determination of the relative proportions of each oxidized monomer. We have, therefore, preferred to regroup these oxidized monomers into 5 groups, as shown in Figure 1, according to their R_X values and, consequently, their polarity. The respective proportions of the 5 groups were then calculated (28) from the corresponding areas determined by planimetry on the photodensitograms. No attempt was made to determine if the same charring intensity/μg of carbon was given by each of these groups. In these conditions, the oxidized monomer composition of the lymphatic lipids in Table I must be considered as relative to that of the dietary lipids. On the contrary, the calculated values of lymphatic recovery certainly were not influenced by such eventual differences on charring. Indeed, for each studied oxidized monomer group, the absorption rate was calculated from the quantities of carbon under the corresponding 2 homolog peaks in the photodensitograms of the lymphatic and dietary lipids.

From the results shown in Table I, a selection seemed to exist between the different oxidized monomers, the lymphatic absorptions of which were between 25 and 93%. Furthermore, with the exception of group I constituents, which only account for 5% of total oxidized monomeric acids in heated soybean oil, the absorption through the lymph seemed to increase with the apolarity of the oxidized derivative. A lymphatic recovery of 16% has been reported (9) for ricinoleic acid. In our TLC conditions, this hydroxy acid would correspond to a group IV compound.

Intestinal Absorption of Polymeric Acids

Figure 2 is a comparison of the photodensitograms obtained after TLC separation of the polymeric derivatives present in heated soybean oil and lymphatic lipids. The chromatographic conditions separate these polymers

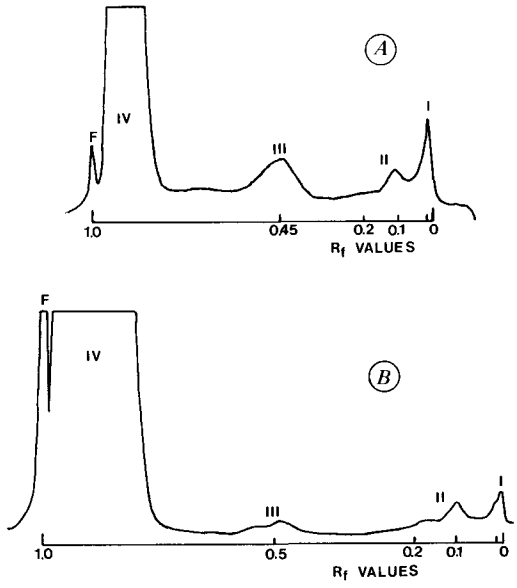

FIG. 2. Photodensitograms of the methyl esters corresponding to polymeric acids (peaks I, II, III) in heated soybean oil (A) and in lymphatic lipids (B). The band IV corresponds to normal and cyclic fatty acid esters present in the sample (see Materials and Methods). F = solvent front.

into 3 classes. Their relative proportions and lymphatic recoveries are shown in Table II. It is necessary to underscore that, to obtain satisfactory photodensitograms for quantitative determinations, the lymphatic lipid sample to be chromatographed was considerably larger than the heated oil sample in order to compensate for the low polymer content in the lymphatic lipid sample. Moreover, the consequences of possible differences in charring densities of these 3 polymer classes were the same as those previously discussed.

In the photodensitograms, peak III certainly corresponded to nonpolar dimeric compounds. First, the R_F value (0.45) was comparable to that of a model thermal dimer in the same solvent (8,30). Second, compound III represented 2% of the total fatty acids in heated soybean oil (Table II). This percentage corresponds to the average nonpolar dimer content present in fats which have been heated in conditions similar to ours (8,35). Finally, the fraction of compound III absorbed through the lymph (1%) was of the same order as that measured (0.2%) with purified nonpolar dimer (12), especially if the fact is taken into account that the purified nonpolar dimer was administered to the rats as methyl ester. The double peak II (R_F values: 0.10 and 0.20) and peak I (R_F: 0.02) in the Figure 2 photodensitograms are components previously identified in the heated oils as dimers and oligomers, both of which are polar (36).

The toxicity observed in certain nutritional studies with heated oils has, in a number of cases, been attributed to the presence of polymeric acids and, more precisely, to their polar constituents (37). Our results show that, in fact, these polar polymers are the most readily absorbed: 6.8 and 12% for polar dimers and polar oligomers, respectively, against 1% for the nonpolar dimers (Table II). On the other hand, however, this higher intestinal absorption is, to a certain degree, compensated by the fact that polar polymers are less abundant in the heated oil than their nonpolar homologs (Table II). This is in agreement with the observation that polar dimers present in a heated oil are only dangerous when the oil is fed to the rats in a sufficiently large quantity, or if the animals ingest the purified polar fraction of the dimeric acids (37). Diarrhea often was described in these cases (12,38). In our study, such a symptom was never observed during the 48-hr test period.

The Intestinal Absorption of Cyclic Monomeric Acids

The cyclic monomers generated during the heating of oils have also been cited as potentially toxic compounds (37). The only available information on their lymphatic absorption shows that 61% of a labeled cyclohexadienyl methyl ester ingested by rats are recovered into the lymph (11). This study compares the absorption of the total cyclic monomers contained in a heated oil and the absorption of 2 model acids, one aromatic, the other cyclohexenic. The results are shown in Table III.

When rats were given heated Primor colza oil, 96% of the total cyclic monomeric acids were absorbed through the lymph. Under our GLC conditions, the cyclic derivatives in the heated oil eluted as 4 major peaks, in agreement with previously published results (32). The absorption percentages calculated for each of these 4 fractions were between 93 and 98%. These particularly high values are in no way

TABLE II

Relative Composition of the Dietary and Lymphatic Polymeric Fatty Acids and Lymphatic Absorption of the Various Polymers

	Total fatty acids (%)		Total polymers (%)		
	Heated soybean oil	Lymphatic lipids	Heated soybean oil	Lymphatic lipids	Lymphatic absorption[a] (%)
Total polymers	4.60	0.14	100	100	4.2
Fraction 1[b] (polar oligomers)	0.46[c]	0.04[c]	10	29	12.0
Fraction 2[b] (polar dimers)	1.60[c]	0.08[c]	35	57	6.8
Fraction 3[b] (nonpolar dimers)	2.54[c]	0.02[c]	55	14	1.0

[a]Calculated using the isotopic dilution factor 1.38 (see Materials and Methods).

[b]The fraction numbers correspond to those indicated on the photodensitograms in Fig. 2.

[c]Calculated compositions. The other compositions were obtained from chromatographic data.

TABLE III

Lymphatic Recovery of Various Dietary Cyclic Monomeric Fatty Acids

Experiment number	Dietary lipid[a]	GLC retention ratio[b]	Total fatty acids (%)		Lymphatic absorption (%)[c]
			Dietary lipids	Lymphatic lipids	
II (9 rats)	Total cyclic monomers	1.06-1.35	3.1	2.0	96
	Component 1	1.06	0.22	0.14	95
	Component 2	1.12	0.64	0.42	98
	Component 3	1.24	1.06	0.69	97
	Component 4	1.35	1.17	0.73	93
III (7 rats)	9-(2′-propylbenzene) nonanoic acid	3.47	0.50	0.18	51
IV (7 rats)	9-(2′-propylbenzene) nonanoic acid	3.47	9.50	3.30	60
V (7 rats)	9-(2′-propyl cyclohexen-1′-yl) nonanoic acid	1.60[d]	0.24	0.14	96
		1.72[d]	1.10	0.61	91
		1.81[d]	2.70	1.60	98
VI (7 rats)	9-(2′-propyl cyclohexen-1′-yl) nonanoic acid	1.81	0.22	0.13	95

[a]Heated Primor colza oil (experiment II) or synthetic triglycerides (experiments III-VI) were fed to the rats. Within each experiment, the lymphs obtained from the rats fed the same fat were combined.

[b]Retention time of component retention:time of methyl stearate; GLC were performed after (exp. II) or without (exp. III-VI) hydrogenation of methyl esters (see Materials and Methods).

[c]Calculated using the following isotopic dilution factors: 1.50 (exp. II), 1.43 (exp. III), 1.74 (exp. IV), 1.65 (exp. V) and 1.61 (exp. VI) (see Materials and Methods).

[d]Respective retention ratios of the 3 cyclohexenyl isomers (see Materials and Methods).

related to the fact that heated colza oil contained large quantities of cyclic monomeric acids (3% of the total fatty acids). As a matter of fact, an identical absorption was measured for 9-(2′-propyl cyclohex-4′-en-1′-yl) nonanoic acid whether it represented 0.3 or 4% of the triglyceride total fatty acids fed to the rats. This result is important insofar as this synthetic acid is an excellent model for alicyclic structures which compose the majority of the cyclic monomeric fraction of heated oils (5). The aromatic acids are absorbed to a lesser degree, judging from the results obtained with the disubstituted benzene model.

In the course of this study, the NVOP lymphatic recoveries were calculated considering that the absorption of [^{3}H] triolein, known to be complete (22), was not reduced by the presence of NVOP in the intestinal lumen. This certainly was true, as suggested by the fact that isotopic dilution factors comparable to our own values may be calculated from experiments in which cannulated rats were fed only triolein (39). Moreover, it must be remembered that in the present work, the NVOP quantities ingested by the animals were low (< 10% of total dietary fatty acids) compared to those of normal and well absorbed fatty acids also present in the dietary heated oils or synthetic triglycerides. Finally, the lymph was collected over 48 hr, whereas absorption of oleic acid was achieved within 24 hr (21). All these conditions are favorable to a complete recovery of the added [^{3}H] triolein.

The results obtained during this study suggested a discrimination exists between the various NVOP at the intestinal mucosa level. This differential absorption could result from several factors. The triglyceride hydrolysis in the intestinal lumen is a prerequisite step in the absorption of fatty acids, either free or as monoglycerides. The pancreatic lipase activity on triglycerides is known to be influenced by the structure of the fatty acids and by their position on the triglyceride skeleton (40). Among the NVOP esterified in the 1- or 3-position of the *sn*-glycerol, it seems likely that the preferential hydrolysis of certain of these compounds could favor their transfer into the lymph as free fatty acids. In this connection, it is not inconceivable that all the dietary NVOP may be recovered in the lymph as monoglycerides, independently of their structure, if they were in the 2-position of ingested triglycerides, provided that pancreatic lipase hydrolyzes the fatty acids in 1- and 3-positions. The distribution of the NVOP on the *sn*-glycerol was, however, unknown in the heated oils and synthetic glycerides used in this work. From another point of view, because of their large structural diversity, certain NVOP, or their corresponding monoglycerides, could be more

soluble than others in bile salt micelles (41,42), thus favoring their intestinal absorption (41). The observed differences in the NVOP lymphatic recovery as a function of their polarity therefore could be significant. Of course, these are hypotheses for which validity studies must be done.

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