Separation of Natural Polyunsaturated Fatty Acids by Means of Iodolactonization

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Conditions of the iodolactonization reaction (IL-reaction) were optimized as a method for separation of natural polyunsaturated fatty acids. The effects of the solvent, temperature and the ratio of components of the iodizing complex KI/I_2 upon the rate of the synthesis of several iodolactones (ILs) in the IL-reaction are described. It was shown that the rate of formation of γ-ILs was significantly higher than that for δ-ILs. This offers opportunity for obtaining pure docosahexaenoic acid (DHA) from fatty acid (FA) concentrates. The possibility for selective reduction of δ-ILs in the presence of γ-ILs to yield pure arachidonic acid (AA) or eicosapentaenoic acid (EPA) or fatty acid concentrates has been demonstrated. Preparation of pure AA from a mixture of AA, DHA and other FAs by the IL-reaction without chromatographic procedures is described.

KEY WORDS: Arachidonic acid, iodolactonization, separation of fatty acids.

The chemistry of the polyunsaturated fatty acids (PUFAs), including arachidonic (AA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids has recently become of interest in biochemical and medical research (1). Particular interest has been focused on the eicosanoids (2), compounds derived through oxidation of PUFAs. Their critical importance in human physiology is increasingly well-documented (3). Detailed study of these relationships will require use of pure PUFAs and dietary mixtures enriched with certain amounts of AA, EPA and DHA. Thus, development of simple methods for separating and purifying PUFAs should be recognized as an urgent problem.

It has been shown that some unsaturated carboxylic acids interact with iodine in alkaline solutions to form iodolactones (ILs) (4). Corey and his co-workers (5) proposed application of the iodolactonization reaction (IL-reaction) for purification of PUFAs from compounds of technical purity. They described synthesis of iodolactones of arachidonic (IL-AA, I), eicosapentaenoic (IL-EPA, II) and docosahexaenoic (IL-DHA, III) acids (6,7) (Scheme 1).

It should be noted that the authors selected conditions for the IL-reaction under which only acids with Δ4 and Δ5 double bonds, i.e., AA, EPA, DHA and some minor acids, yield iodolactones. These can be separated from unreacted PUFAs and then reduced to yield the original acids. Thus, the procedure affords a mixture of AA, EPA and DHA, their proportion depending on the fatty acid composition of the starting material.

With controlled conditions for the IL-reaction, one can obtain pure DHA from fish oil (8). At the same time, the authors note that selection of optimal conditions for separating one acid from the initial mixture requires considerable experimental work.

In this connection, we have planned and conducted a comparative study of the rates of formation of ILs of different structures (I, II and III) and their reduction to initial PUFAs, depending on reaction conditions.

MATERIALS AND METHODS

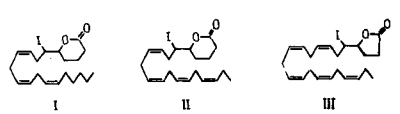
Total fatty acids of sardine oil and rat liver were obtained by saponification of total lipids according to Kates (9). Enrichment of PUFAs of marine origin was carried out by crystallization from acetone for 24 hr at −25°C. Potassium iodide, I_2, $KHCO_3$, dioctylphthalate, methylstearate and pentadecanoic acid of high purity were used in the reaction. TMS-iodide was obtained from Far East State University (Vladivostok, USSR). Benzene and acetonitrile were distilled over P_2O_5, other solvents were purified by distillation.

We used high performance liquid chromatography (HPLC) on a Yanaco L-2000L chromatograph (Yanaco Ltd., Kyoto, Japan) with a Separon RP18 column, 5 mm, 4 × 150 mm (Tessek Co., Praha, Czechoslovakia). Detection at 254 and 230 nm was conducted while using a $MeOH/H_2O$ (85:15) solvent system (1 mL/min).

Gas-liquid chromatography (GLC) of FAs was performed in a Biochrom 1 (NPO Manometer, Moscow, USSR) instrument with a 4.5 m × 3 mm column, liquid phase 3.5% FFAP on Chromaton N-AW-HMDS, 100–125 μm, at a temperature of 210°C, and the carrier gas was helium (40 mL/min). FAs were identified by comparison with authentic standards. Methyl esters of fatty acids were prepared for GLC according to Kates (9).

Thin-layer chromatography (TLC) was conducted according to the method described earlier by Svetashev and Vaskovsky (10). FAs and their esters were analyzed with TLC in n-hexane/ether/acetic acid, 80:20:1. Column chromatography was performed on silica gel G (40–80 μm), separation of iodolactones was checked with TLC in pure benzene: R_f 0.29 for IL-AA (IL-EPA) and R_f 0.35 for IL-DHA. UV- and mass-spectra obtained for ILs (I, II, III) corresponded to those given in the literature (7,8).

Kinetics of iodolactonization. Enriched sardine oil



I II III

SCHEME 1

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FAs (400 mg) (29.9% EPA, 37.5% DHA) were suspended in 3.1 mL of 16% aqueous $KHCO_3$ and 0.5 mL organic solvent (tetrahydrofuran, 1,4-dioxane or 96% ethanol). Forty mg of dioctylphthalate in 0.4 mL of organic solvent was added. A water solution of KI (1 g/mL) was added to obtain the required concentration (Figs. 1 and 2) and the mixture was then cooled to experimental temperature. The required amount of iodine solution in 5 mL of appropriate organic solvent was added dropwise (Figs. 1 and 2). A portion of the reaction mixture (0.5 mL) was taken for assay after 1, 5, 10, 15, 30, 45, 60, 120, 180, 240 and 300 min, mixed with concentrated $Na_2S_2O_3$ solution (1 mL) and extracted with 0.8 mL benzene. The upper layer was passed through a microcolumn with 0.5 g of silica gel, and the ILs were eluted with 5 mL of 20:1 benzene/ethylacetate, then evaporated, redissolved in 1 mL of MeOH and analyzed by HPLC. The amounts of IL-EPA (k′ = 3.43) and IL-DHA (k′ = 4.83) were identified by using dioctylphthalate (k′ = 11.0) as an internal standard.

Linoleic acid in the IL-reaction. Linoleic acid (40 mg) and pentadecanoic acid (0.1 mg) (internal standard) were suspended in 16% $KHCO_3$ (8 mL), 96% ethanol (2 mL), KI water solution (652 mg/mL) and I_2 (426 mg in 5 mL of ethanol). A 0.5-mL aliquot portion of the mixture was taken for analysis. Excess iodine was neutralized with $Na_2S_2O_3$, and 1 n HCl was added to obtain pH 2. FAs were extracted with hexane and converted to methyl esters for analysis by GLC. KI was not added in the control test.

Reduction of IL mixture with different molar ratios TMS-iodide/ILs. Five mg of IL-AA and IL-DHA mixture, 4:6 (mole), were dissolved in 0.2 mL of dry acetonitrile, and different amounts (0.1 to 5.0 eq.) of TMS-iodide in acetonitrile were added dropwise under argon. After 20 min exposure at 23°C, 0.5 mL of 5% HCl in MeOH and methylstearate in C_6H_6 (0.5 mg/0.25 mL) were added and incubated during 15 min at 50°C. The methyl esters were extracted with hexane, purified with preparative TLC in benzene (R_f 0.85) and analyzed with GLC. A composition of FAs was calculated in terms of methylstearate.

Separation of arachidonic acid. Enriched FAs (1 g) from rat liver (22.2% of AA; 0.8% of EPA; 2.9% of 22:5ω6; 5.95% of DHA) were dissolved in 3 mL of ethanol and 7.5 mL of 16% aqueous $KHCO_3$. A mixture of 89 mg KI and 68 mg I_2 in 11 mL of ethanol was added. After 20 hr at 10°C in the dark, the reaction mixture was extracted with 3 × 10 mL of 2:1 hexane/ether, and 352 mg KI and 741 mg I_2 were added to the water layer. After 6 hr at 15°C in the dark, the iodine was neutralized by aqueous 40% $Na_2S_2O_3$. Twenty mL of water was added and the mixture was extracted with 2 × 30 mL 4:1 hexane/ether. The extract was evaporated, redissolved in 20 mL of hexane and washed with 3 × 15 mL of 6% $KHCO_3$ solution in 50% water/ethanol. The organic layer was evaporated and redissolved in 2 mL of dry acetonitrile, and 0.36 mL of TMS-iodide in dry acetonitrile (0.5 mL/2 mL) was added dropwise. After 10 min, iodine was neutralized with $Na_2S_2O_3$ solution and FAs were extracted with 2 × 16 mL hexane/ether (2:1). The organic layer was evaporated to yield 92 mg of FAs composed of 97.8% of AA and 1.6% of EPA.

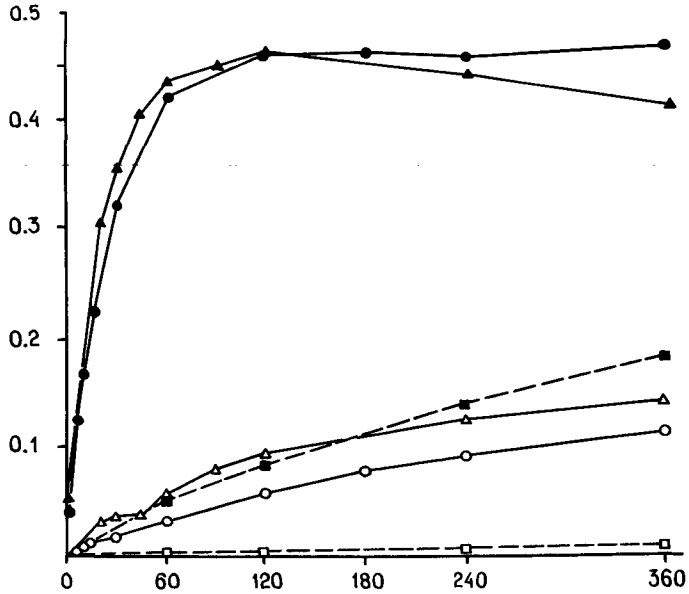

FIG. 1. Synthesis of iodolactones of eicosapentaenoic (IL-EPA) and docosahexaenoic (IL-DHA) acids in different organic solvent systems. Five hundred mg KI and 400 mg I_2 were used for 400 mg of fatty acids (containing 0.397 mmoles EPA and 0.457 mmoles DHA) at 13°C (cf. Materials and Methods). Dioxane/H_2O; IL-DHA, ●——●; IL-EPA, ○——○; ethanol/H_2O; IL-DHA, ▲——▲; IL-EPA, △——△; and tetrahydrofuran/H_2O; IL-DHA, ■----■; IL-EPA, □----□.

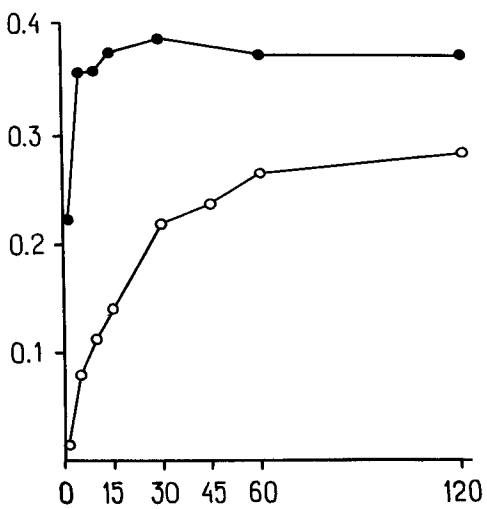

FIG. 2. Synthesis of iodolactones of eicosapentaenoic (IL-EPA) and docosahexaenoic (IL-DHA) acids in ethanol at 25°C. Five hundred mg KI and 400 mg I_2 were used for 400 mg of fatty acids (containing 0.397 mmoles EPA and 0.457 mmoles DHA). IL-DHA, ●——●; and IL-EPA, ○——○.

RESULTS AND DISCUSSION

Starting reaction conditions for the IL-reaction were

TABLE 1

Synthesis of Iodolactones of EPA (IL-EPA) and DHA (IL-DHA) under Varying Proportion of KI/I_2[a]

Reaction time (min)	KI/I_2, mole/mole							
	0		0.5		1.0		2.0	
	IL-DHA	IL-EPA	IL-DHA	IL-EPA	IL-DHA	IL-EPA	IL-DHA	IL-EPA
1	0.208[b]	0.114	0.364	0.088	0.063	—	0.093	—
5	—	0.172	0.320	0.184	0.292	0.031	—	—
10	0.153	0.203	0.336	0.233	0.330	0.049	0.286	0.032
15	—	—	0.321	0.251	0.348	0.062	—	—
20	0.149	0.225	—	—	—	—	0.354	0.053
30	0.147	0.227	0.337	0.283	—	—	0.374	0.068
40			—	—	0.343	0.122	—	—
60			0.333	0.276	—	—	0.369	0.119
90					0.339	0.212	—	—
150					—	—	0.371	0.206
180					0.324	0.267	—	—
300							0.371	0.261

[a]2.56 mmoles of I_2(652 mg) were used for the mixture of fatty acids (400 mg) containing 0.397 mmoles of EPA and 0.457 mmoles of DHA (organic solvent = ethanol, 20°C; cf. Materials and Methods).
[b]All data expressed as amount of iodolactones (mmoles).

chosen as described in (5). It was known that γ-lactones are more stable and are produced under milder conditions than the δ-lactones. The same was also expected for δ-IL-AA (I), δ-IL-EPA (II) and γ-IL-DHA (III); in practice, the rate of IL-DHA (III) synthesis was significantly greater than that for IL-EPA (II) (Fig. 1). The rate of IL synthesis is highly dependent on the nature of the solvent. As Figure 1 shows, 95% of DHA and only about 10% of EPA reacted in ethanol during 1 hr. Apparently, the reaction could be stopped at a predetermined moment in order to obtain, after reduction of ILs, an end product with the desired proportion of EPA and DHA. Tetrahydrofuran is recommended as solvent for synthesis of pure IL-DHA, the reaction requiring about 20 hr. In dioxane the reaction generally follows the same pathway as in ethanol. It should be noted that in large-scale experiments ethanol is preferable as a solvent, because, in contrast to dioxane, the use of ethanol makes it possible to reach considerably greater concentrations of iodine reagent in the reaction mixture.

The rate of IL synthesis is highly temperature-dependent. Figure 2 gives the parameters of IL-reaction in ethanol, conditions being the same as for Figure 1, but at a temperature of 25°C. The amount of IL-DHA (III) reaches a maximal value in 10 min, while that of IL-EPA (II) requires 1.5 hr. The amount of IL-DHA decreased by 13–15% (compared to that obtained at 13°C).

The limiting stage of the method proposed for PUFAs production is IL synthesis. The reaction was shown to require 8–48 hr (7,8). We have observed that the rate of this reaction depends not only on I_2 excess, but specifically on the relationship KI/I_2 (the complex KI_3 is built) (Table 1). It is clear that in the absence of KI, addition of I_2 leads to iodolactone synthesis practically immediately, but half of the DHA does not participate in the reaction and is irreversibly lost. With addition of 0.5 equivalent of KI, the reaction rate remains high and is completed in 30 min. In both cases, the presence of free I_2 leads later to decrease in the IL content. An equivalent ratio of KI/I_2 protects free fatty acids and synthesizes ILs without undesired side-reactions. IL-DHA and IL-EPA synthesis require 15 min and 3 hr, respectively. A double excess of KI only slightly diminishes the rate of γ-lactone (III) synthesis, while it inhibits the rate of δ-lactone (II) synthesis. In the IL-reaction without KI, iodine should be used with care because of consequent rapid PUFA destruction. Mono-, di- and trienoic FAs are also subject to destruction. Linoleic acid does not form IL under these reaction conditions, but its concentration in the KI-free reaction mixture decreases rapidly. The use of equivalent KI/I_2 completely inhibits this process (Table 2).

TABLE 2

Stability of Linoleic Acid in the IL-Reaction. The Yield of Linoleic Acid (mg) is Shown

Iodine reagent	IL-reaction time (min)				
	0	5	15	30	60
I_2	400	97	82	64	76
KI_3	400	378	400	381	397

Thus, using different ratios of KI/I_2, one can obtain either the total EPA and DHA (30 min, KI/I_2 = 0.5), or a concentrate, considerably enriched with DHA (30 min, KI/I_2 = 1.5) (data not provided). Since γ-IL is presumably the end product in any case, it is evident that with tetrahydrofuran we obtain practically only IL-DHA (Fig. 1), which can be used to prepare pure DHA. Wright, Kuo and Corey (8) described this IL-synthesis under conditions where DHA/I_2 = 1.2 and KI/I_2 = 1. Other fatty acids with Δ4 double bonds (for example, 22:5ω6) may form γ-IL similar to DHA.

In contrast to DHA, synthesis of AA and EPA

requires another approach. The study of the IL reduction to FA esters with TMS-iodide has made it possible to obtain concentrates enriched with AA (or EPA) or, with somewhat lower yields, these acids in pure form. Figure 3 presents the dependence of the reduction rate of a mixture of δ- and γ-IL upon the amount of reducing agent TMS-iodide added. It can be seen that, with insufficient TMS-iodide, 80% of IL-AA reduced without apparent synthesis of DHA ester from IL-DHA component of the mixture. It follows that: i) selective reduction of δ-IL in the presence of γ-IL is possible; ii) 1.2–1.5 eq. of TMS-iodide is required for complete reduction of the IL mixture; iii) with more than 3–5 eq. of TMS-iodide applied, reaction yield falls sharply; and iv) desired ratios of AA (EPA) and DHA in the product can be obtained in preparative reductions of IL mixtures by use of varying amounts of TMS-iodide. Sodium iodide and TMS-chloride can also be used for IL reductions.

As we have noted above, pure DHA can be obtained from a mixture of FA containing AA and EPA (8) without chromatographic separation of δ- and γ-ILs. This is possible because of the higher rate of γ-IL synthesis compared to that for δ-IL synthesis (Figs. 1 and 2). In order to illustrate our concept of the range of application of the iodolactonization method, we made an attempt to separate AA without chromatographic separation of ILs from a rat liver FA mixture containing DHA and 22:5ω6 acid (its conversion to γ-ILs). To this end, the IL-reaction was carried out at molar ratios $DHA/I_2 = 1$ and $KI/I_2 = 2$. Under these conditions, IL-AA does not form in significant amount, and the IL-DHA product was removed from the reaction mixture by extraction. The reaction was continued with a new portion of I_2 ($AA/I_2 = 0.25$, $KI/I_2 = 1$) added. The IL-AA and other lactones produced were reduced by means of insufficient amount of TMS-iodide (IL-AA/TMS-iodide = 0.9). As a result, we obtained 97% AA with an admixture of 2% EPA and 1% of minor fatty acids.

We should note another important advantage of the method of iodolactonization. The intermediate iodolactones can be used readily for synthesis of corresponding hydroxy-acids, products of the lipoxygenase pathway of FA oxidation, which are powerful natural biostimulators. In this way, IL-AA, IL-DHA and IL-EPA were used for the synthesis of 5-hydroxyeicosatetraenoic acid, 4-hydroxydocosahexaenoic acid and 5-hydroxyeicosapentaenoic acid by the method of Corey *et al.* (6).

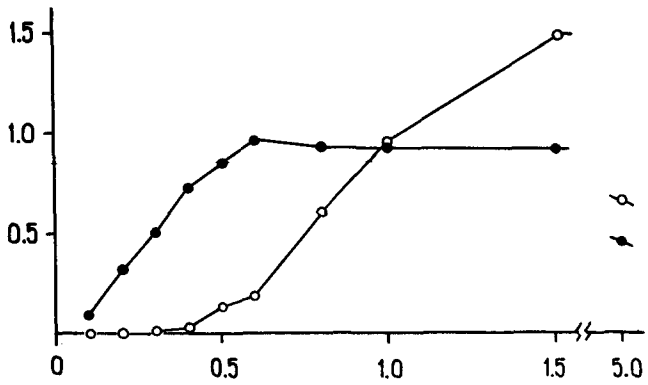

FIG. 3. Reduction of a mixture of iodolactones of docosahexaenoic and arachidonic acids to the corresponding FA methyl ester, with reference to TMS-iodide amount added. Methyl ester of docosahexaenoic acid, ○——○; and methyl ester of arachidonic acid, ●——●.

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