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OXYGENATION OF LINOLENIC AND LINOLEIC ACID TO NOVEL VICINAL DIHYDROXY ACIDS BY HEPATIC MICROSOMES OF THE RABBIT

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SUMMARY: [^{14}C] Linolenic acid (18:ω3) and [^{14}C] linoleic acid (18:2ω6) were incubated with hepatic microsomes of the rabbit in the presence of NADPH (1 Mm) for 15 min at 37°C. The products were extracted and purified by high performance liquid chromatography. The major metabolites of linolenic and linoleic acid were identified by capillary gas chromatography mass spectrometry as 15,16-dihydroxy-9,12-octadecadienoic acid, 12,13-dihydroxy-9,15-octadecadienoic acid and 9,10-dihydroxy-12,15-octadecadienoic acid and as 12,13-dihydroxy-9-octadecaenoic acid and 9,10-dihydroxy-12-octadecaenoic acid, respectively. The results were confirmed by comparison with mass spectra of the authentic compounds. These metabolites are presumably formed by cytochrome P-450 catalyzed epoxidation of the ω3, ω6 and ω9 double bonds, followed by enzymatic hydrolysis to 1,2-diols. The ratio of ω3, ω6 and ω9 oxygenated metabolites of linolenic acid was approximately 2:1:1 and the ratio of the ω6 and ω9 metabolites of linoleic acid was 2:1, indicating that the double bond closest the ω end is most easily oxygenated.

Linolenic and linoleic acid are important dietary fatty acids and occur in certain plants and seeds. In 1929, GO Burr and MM Burr described a deficiency state in rats fed a fat-free diet (1,2). The syndrome was ameliorated by supplementing the diet with essential fatty acids (EFA). Linoleic and linolenic acid are important polyunsaturated fatty acids of the ω6 and ω3 EFA series and their metabolism has been studied extensively (3,4). In mammals, these two fatty acids are partly desaturated and elongated for form derived EFA (20:3ω6, 20:4ω6 and 20:5ω3), which are precursors of prostanoids and leukotrienes, compounds of great biological interest.

Arachidonic acid (20:4ω6) is one of the most abundant ω6-EFA in mammals. Recent studies showed that arachidonic acid is metabolized to vicinal di-

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hydroxy acids by purified cytochrome p-450 ($PB\text{-}B_2$ and LM_2) and epoxide hydrolase in a reconstituted system as well as by isolated hepatic and renal cells (5-9). It seemed to be of interest to determine whether other EFA are oxygenated in the same way. In the present report, the metabolism of linolenic and linoleic acid by liver microsomes is investigated and the major metabolites, which were formed in the presence of NADPH, were indentified by capillary GC-MS.

METHODS AND MATERIALS

[1-^{14}C] Linolenic acid (18:3ω3) and [1-^{14}C] linoleic acid (18:2ω6), both 56 mCi/mmol, were from Amersham. Linoleic and linolenic acid were from Supelco. m-Chloroperoxybenzoic acid was from Aldrich. BSTFA (bis(trimethylsilyl)trifluoroacetamide) was from Supelco. Glass plates, precoated with silica gel 60 (.25 mm) and most other chemicals were from Merck. Equipment for HPCL was from Laboratory Data control and the following columns were used (Waters and associates): octadecasilane bounded to 10 μm silica gel (μBondapak/C_{18}; 7.8 x 300 mm) and 10 μm silica gel (μPorasil; 3.9 x 300 mm). HPCL solvents were from Rathburn. Methyl 15,16-dihydroxy-9,12-octadecadienoate, methyl 12, 13-dihydroxy-9,15-octadecadienoate, methyl 9,10-dihydroxy-12,15-octadecadienoate, methyl 12,13-dihydroxy-9-octadecaenoate and methyl 9,10-dihydroxy--12-octadecaenoate (all threo) were synthetized and characterized by GC-MS as described (10).

Experimental: Male white New Zealand rabbits (usually 2-3 kg) were treated with phenobarbital (15 mg/kg s.c. x 2 for three days) and were killed on the next day by a blow on the head. Microsomes were prepared from a 20 % liver homogenate as described (8) and resuspended so that 1 ml microsomal suspension corresponded to microsomes from 0.4 g hepatic tissue. The microsomes were used immediately after preparation. The microsomal suspensions (10 ml) were incubated with 1 mM NADPH and the fatty acid (0.18 mM, added in 50 μl ethanol) for 15 min at 37°C under normal atmosphere. An aliquot of the microsomal suspension (0.5 ml) and NADPH were incubated with [1-^{14}C] labelled fatty acid (1-2 μCi) in the same way to ensure radiolabelled metabolites and the two incubations were combined at the end. Control experiments were performed without NADPH. Proteins were precpitated with methanol (4-5 mol) and spun down. The pellet was washed once with methanol. After evaporation, the residue was dissolved in water and extracted twice with ethyl acetate at pH 3. The organic layers were combined, washed with water and dried over Na_2SO_4. After evaporation, the residue was dissolved in methanol-water, centrifuged and the clear supernatant was purified by reversed phase HPCL (μBondapak/C_{18}; 73 methanol 27 water and 0.2 acetic acid; by vol.; flow 2 ml/min/fraction). The metabolites, which were analyzed by GC-MS, were furhter purified as methyl esters by normal phase HPCL (μPorasil; a linear gradient of 20 to 60 % ethyl acetate in hexane in 80 min; flow 1 ml/min/fraction).

Derivatives: An ethereal solution of freshly prepared diazomethane was used for methylation. Silylation was performed with 10 μl BSTFA in pyridine (1:1) for about 10 min at 70° and the samples were then evaporated to dryness under nitrogen and dissolved in n-hexane or n-nonane.

GC-MS analysis: The analyses were performed on a quadropole mass spectrometer (Finnigan 4000) equipped with a data system (Finnigan Incos). The electron energy was 70 eV and the temperature of the ion source set to 300°C.

To increase the sensitivity in the upper mass range, the instrument was calibrated with FC 43 so that the intensity of m/z 69 was 60 %, m/z 219 100 % and m/z 502 10 %. A wall-coated, open capillary GC column of fused silica (25m,OV-101, Hewlett Packard) was used. The samples (in hexane or nonane) were applied by the Grop injection technique (at 50 or 115°C, respectively) and the temperature of the column raised by 20°C/min to 270°C. C values were determined from the retention times of fatty acid methyl esters (C_{18} - C_{24}).

UV-spectrometry: Absorption was measured at 320-200 nm in a Shimadzu 210 A spectrometer using methanol as solvent.

RESULTS

[^{14}C]Linolenic and [^{14}C] linoleic acid were metabolized to many polar metabolites by liver microsomes of phenobarbital-treated rabbits in the presence of NADPH as judged from reversed phase HPLC. Virtually no conversion was noted without this cofactor.

Linolenic acid: A reversed phase chromatogram, which shows the elution of radiolabelled metabolites is shown in Fig. 1. Three peaks of radioactivity eluted (peak I:12-20 ml; peak II:28-32 ml; peak III:44-50 ml). The material

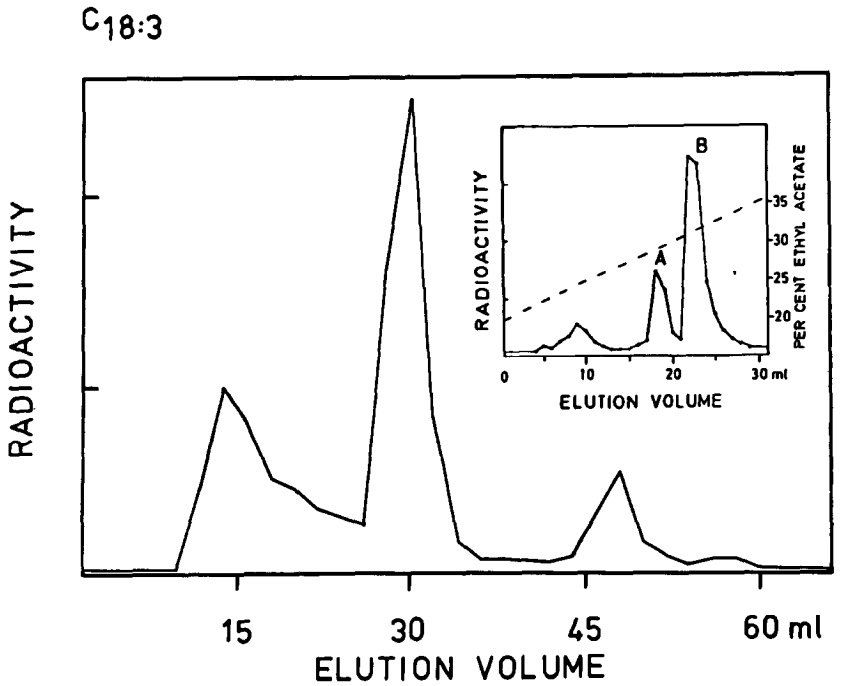

Fig. 1. A reversed phase chromatogram of metabolites formed by an incubation of linolenic acid with hepatic microsomes and NADPH. The insert shows that the major peak could be separated into peak A and peak B by normal phase HPLC.

in peak II was methylated and separated into two components by straight phase HPLC (insert in Fig. 1). The major component (peak B) eluted with 22-24 ml and the minor one with 18-19 ml (peak A). They were subject to GC-MS analysis as TMS ether methyl ester derivatives.

Peak A: A mass spectrum is shown in Fig. 2. The C-value was 21.1. Signals were noted, inter alia, at m/z 470 (M^+), 455 (M^+-15), 439 (M^+-31), 401 (M^+-69) 380 (M^+-90), 349 (M^+-(90+31)), 311 (401-90), 299, 273, 183 (273-90), 171 and 73 (not shown). The fragmentation is proposed by the insert in Fig. 2B. The signal at m/z 270 is presumably due to a rearrangement ion (formed by cleavage between C-11 and C-12 with a transfer of TMS to the carbonyl (10,11)). This mass spectrum was almost identical with that of authentic 12,13-dihydroxy- 9, 15-octadecadienoic acid. Assuming that the double bonds remain in their original positions, the compound in peak A was identified as this 1,2-diol.

Peak B: The GC-MS analysis showed that this peak contained two metabolites, which were separated on GC. The major metabolite had C value 22.0 and the mass spectrum is shown in Fig. 2A. Strong signals were noted at m/z 470 (M^+), 455 (M^+-15), 439 (M^+-31), 380 (M^+-90), 351 (M^+--(29+90)), 339, 249, (339-90), 233, 131 and 73 (not shown). The fragmentation is shown by the insert in Fig. 2A. The strong signal at m/z 310 is presumably formed by rearrangement (cf. m/z 270 discussed above). Assuming that two double bonds remain in their original positions, as indicated by UV-analysis, the major metabolite was identified as 15,16--dihydroxy-9,12-octadecadienoic acid. The mass spectrum of the authentic material was similar except for unimportant differences in relative intensities.

The minor component of peak B had C value 21.0. The mass spectrum is shown in Fig. 2C. Strong signals were noted at m/z 470 (M^+), 455 (M^+-15), 439 (M^+-31), 380 (M^+-90), 361, 349 (M^+-(90+31)) 271 (361-90), 259, 211 and 73 (not shown). The fragmentation is shown by the insert in Fig. 2C. The mass spectrum was very similar to that of the authentic material.

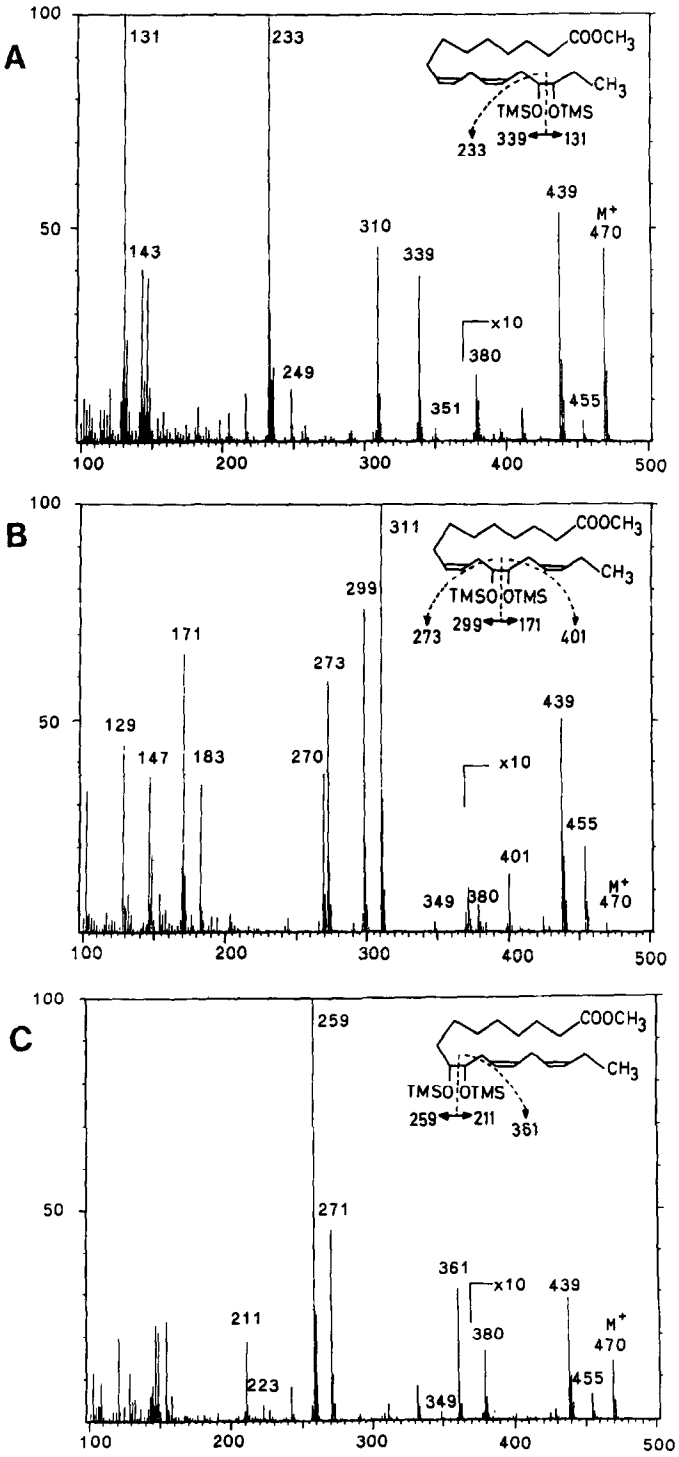

Fig. 2. Mass spectra of three metabolites of linolenic acid (methyl ester trimethylsilyl ether derivatives). A. 15,16-Dihydroxy-9,12-octadecadienoic acid. B. 12,13-Dihydroxy-9,15-octadecadienoic acid. C. 9,10-Dihydroxy-12,15-octadecadienoic acid.

Assuming that two double bonds were unchanged, as indicated by UV-analysis, this metabolite was identified as 9,10-dihydroxy-12,15-octadecadienoic acid.

The three metabolites were estimated to be formed in a ratio of 2:1:1 with 15,16-dihydroxy-9,12-octadecadienoic acid being the major metabolite.

Linoleic acid: The metabolites were separated by reversed phase HPCL into several groups of different polarity. The most polar metabolites eluted with 14-22 ml eluent, the metabolites of medium polarity with 34-38 ml and the least polar metabolites with 48-70 ml. The metabolites of medium polarity which comprised about a third of the recovered [^{14}C] labelled metabolites, were methylated and further purified by straight phase HPCL. Two prominent peaks eluted, a major peak with 20-22 ml (peak C) and a minor one with 23--25 ml (peak D). UV-analysis indicated that these metabolites did not contain conjugated double bonds. The material in peak C and peak D was analyzed by GC-MS (TMS ether methyl ester derivatives).

Peak C: A mass spectrum, which was obtained at C value 21.1 showed the following prominent signals: m/z 472 (M^+), 457 (M^+-15), 441 (M^+-31), 401 (weak signal, presumably M^+-71,loss of carbons 14-18),382 (M^+-90), 311 (401-90), 299 (cleavage between C-12 and C-13, fragment containing C-1 to C-12), 275 (cleavage between C-11 and C-12), 270 (rearrangement), 185 (275-90), 173 (cleavage between C-12 and C-13) and 73. The mass spectrum was almost identical with that of authentic 12,13-dihydroxy-9-octadecaenoic acid and the material of peak C was identified as this compound.

Peak D: The C value was 21.0. The mass spectrum showed signals, inter alia, at m/z 472 (M^+), 457 (M^+-15), 441 (M^+-31), 382 (M^+-90), 361 (M^+-111, loss of C-11 to C-18), 351 (M^+-(90+31)), 271 (361-90), 259 (cleavage between C-9 and C-10, fragment with C-1 to C-9), 213 (cleavage between C-9 and C-10) and 73. 9,10-Dihydroxy-12-octadecaenoic acid showed a very similar mass spectrum and the material in peak D was identified as this 1,2-diol. Peak D contained half the radioactivity of peak C, indicating that about twice as much 12,13-dihydroxy-9-octadecaenoic acid was formed.

that, indeed, phosphodiesterase-I (but not form II) is activated by Ca^{2+}/calmodulin. The apparent molecular weight of the activatable form I enzyme was very similar to that of the non-activatable form I enzyme seen in Fig. 1. In Fig. 2A the calcium activation is evident in head extract of dunce^{M11} flies, which contain only phosphodiesterase-I. Fig. 2B and C show the profiles from larval brain extracts of Canton-S and dunce^{M11} strains, respectively. The poor resolution with Canton-S is due to the high flow rate and resembles the sucrose density patterns obtained by others (5). Nevertheless, it is clear that two phosphodiesterases are present in the brains of wild type larvae and that only form I is activated by calcium. Furthermore, the larval brain phosphodiesterase-I of dunce^{M11} is equally active toward cyclic GMP, like the enzyme from whole animals (1,2), and cyclic GMP hydrolysis is also enhanced by Ca^{2+}/calmodulin.

While completing this manuscript, we noticed the very recent paper by Kauvar (14) on Drosophila phosphodiesterases. He has detected a labile form III enzyme that is specific for cyclic GMP. More importantly, in the present context, he also observed the loss of Ca^{2+}-activatability, described above, but attributed it to irreversible activation by proteolysis. We observed a decrease, rather than an increase, of basal activity during aging of the form I enzyme (Table 1). The source of this discrepancy is not yet clear: possibly an irreversible activation (by Ca^{2+}-desensitization) was superimposed on an inactivation under our conditions.

Our demonstration of the two forms (I and II) of cyclic nucleotide phosphodiesterase in the actual brain tissue of Drosophila melanogaster larvae encourages attempts to define the exact ultrastructural location of the form II enzyme within the insect's nervous system, in view of the impaired learning ability of Drosophila mutants lacking this enzyme.

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parison with the three 1,2-diols. By analogy with the microsomal metabolism of arachidonic acid, the nonpolar metabolites could contain trivial monohydroxy acids, formed by ω- and (ω-1)-hydroxylation or peroxidation, while the most polar metabolites could contain trihydroxy acids, formed by a combination of epoxidation and hydroxylation reactions (6).

It will be of interest to determine whether epoxidation of linolenic and linoleic acid occurs in vivo.

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