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Enzymatic Studies on the Mechanism of the Retroconversion of C_{22}-Polyenoic Fatty Acids to their C_{20}-Homologues

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Summary: The retroconversion of specifically labelled C_{18}-, C_{20}- and C_{22}-polyenoic fatty acids ($18:2^{9,12}$; $20:2^{11,14}$; $20:3^{8,11,14}$; $20:4^{5,8,11,14}$; $22:3^{10,13,16}$; $22:4^{7,10,13,16}$) to their (*n*-2) bishomologues has been studied *in vivo* and *in vitro*. Only the docosapolyenoic acids were retroconverted to the corresponding eicosapolyenoic acids. The partial degradation was demonstrated in experiments *in vivo* and *in vitro* with rat liver subcellular fractions.

The C_{22}-polyenoic acids and their chain shortened C_{20}-derivatives were found to be distributed in the experiments *in vivo* in all cell fractions, mitochondria, microsomes and supernatant as well. However studies *in vitro* proved that only the mitochondrial fraction is capable of catalyzing the retroconversion of the C_{22}-polyenoic fatty acids to their C_{20}-homologues. Therefore the chain shortening reaction is not due to a reversed chain elongation but to a partial β-oxidation. Further proof for a β-oxidation process was obtained by using submitochondrial fractions which were characterized by their marker enzymes and three β-oxidation enzymic activities: Enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and Δ^{3cis}-Δ^{2trans}-enoyl-CoA isomerase. These enzymes, together with the enzymes involved in the retroconversion reactions of the docosapolyenoic acid, are localized on the inner mitochondrial membrane. The chain shortening reactions in the mitochondria proceed unidirectionally. Exchange reaction with acetyl-CoA and malonyl-CoA did not take place.

Dihydroxyacetone phosphate and glycerol 3-phosphate were potential acceptor molecules for the substrate, the docosapolyenoic acid and the product, the eicosapolyenoic fatty acid.

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Enzymes:

Acyl-CoA synthetase, acid:CoA ligase (AMP) (EC 6.2.1.3) (= thiokinase)

Cytochrome oxidase, ferrocytochrome *c*: oxygen oxidoreductase (EC 1.9.3.1)

Enoyl-CoA hydratase, L-3-hydroxyacyl-CoA hydro-lyase (EC 4.2.1.17) (= Crotonase)

Δ^{3cis}-Δ^{2trans}-Enoyl-CoA isomerase (EC 5.3.3.? not yet listed)

Glucose-6-phosphatase, D-glucose-6-phosphate phosphohydrolase (EC 3.1.3.9)

3-Hydroxyacyl-CoA dehydrogenase, L-3-hydroxyacyl-CoA:NAD oxidoreductase (EC 1.1.1.35)

3-Hydroxybutyryl-CoA epimerase, 3-hydroxybutyryl-CoA 3-epimerase (EC 5.1.2.3) (= D(−)3-hydroxy-acyl-CoA 3-epimerase)

Malate dehydrogenase, L-malate:NAD oxidoreductase (EC 1.1.1.37)

Monoamine oxidase, monoamine:oxygen oxidoreductase (deaminating) (EC 1.4.3.4)

Phospholipase C, phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3).

Zusammenfassung: *Enzymatische Untersuchungen zum Mechanismus der Rückverwandlung von C_{22}-Polyenfettsäuren in ihre C_{20}-Homologe.* Die Rückverwandlung von spezifisch markierten C_{18}-, C_{20}- und C_{22}-Polyenfettsäuren ($18{:}2^{9,12}$, $20{:}2^{11,14}$, $20{:}3^{8,11,14}$, $20{:}4^{5,8,11,14}$, $22{:}3^{10,13,16}$, $22{:}4^{7,10,13,16}$) zu ihren um 2 C-Atome verkürzten Bishomologen wurde in vivo und in vitro untersucht. Nur die Docosapolyenfettsäuren wurden zu den entsprechenden Eicosapolyensäuren abgebaut. Dieser partielle Abbau konnte in vivo und im In-vitro-Experiment mit subzellulären Fraktionen der Rattenleber gezeigt werden.

Im In-vivo-Versuch wurden die C_{22}-Polyensäuren und ihre kettenverkürzten C_{20}-Derivate in allen Zellfraktionen verteilt aufgefunden. Die Versuche *in vitro beweisen jedoch, daß nur die mitochondriale* Fraktion in der Lage ist, die Kettenverkürzung zu katalysieren. Die Kettenverkürzung ist daher nicht eine Umkehr der am endoplasmatischen Reticulum ablaufenden Kettenverlängerung, sondern eine partielle β-Oxidation. Ein weiterer Beweis für einen β-Oxidationsprozeß stellten Versuche mit submitochondrialen Fraktionen dar, die durch ihre Leitenzyme und die folgenden Enzymaktivitäten *der β-Oxidation charakterisiert wurden:* Enoyl-CoA-Hydratase, 3-Hydroxyacyl-CoA-Dehydrogenase, $\Delta^{3\,cis}$-$\Delta^{2\,trans}$-Enoyl-CoA-Isomerase. Diese Enzyme zusammen mit den für die Kettenverkürzung verantwortlichen sind auf der inneren Mitochondrienmembran lokalisiert. Die Kettenverkürzung im Mitochondrion verläuft unidirektional. Ein Austausch in Gegenwart von Acetyl- und Malonyl-CoA konnte nicht beobachtet werden. Dihydroxyacetonphosphat und Glycerin-3-phosphat *sind Akzeptormoleküle für das Substrat, die* Docosapolyenfettsäure und das Produkt, die entsprechende Eicosapolyensäure.

The biosynthesis of polyunsaturated fatty acids in the mammalian cell has been studied extensively[1–6]. Both the chain elongation and desaturation reactions have been achieved in experiments *in vitro using subcellular fractions of rat liver.* The enzyme systems involved are localized in the membranes of the endoplasmic reticulum. Substrates of the chain elongating enzymes are the long chain acyl-CoA derivatives and malonyl-CoA. NADPH serves as the donor of the reduction equivalents required. The coenzyme A derivative of the long chain fatty acids is also the substrate for the microsomal desaturase system and molecular oxygen is required for the introduction of the *cis*-double bonds in the carboxy terminal part of the substrate.

An acetyl-CoA dependent chain elongating system localized in the mitochondria of rat liver, rabbit and beef heart has been described[7–13]. Its biological importance for the biosynthesis of C_{20}- and C_{22}-polyenoic fatty acids compared with the very active microsomal system is questionable, particularly in view of the chain length of the fatty acids synthesized and also in comparison with the very active microsomal malonyl-CoA requiring system. It has been suggested, that the mitochondrial chain elongation is operative in a reversed one or two step β-oxidation sequence.

The β-oxidation of mono- and polyunsaturated fatty acids has also been unravelled by experiments *in vitro.* Two additional mitochondrial enzymes, the $\Delta^{3\,cis}$—$\Delta^{2\,trans}$-enoyl-CoA isomerase and D(-)3-hydroxyacyl-CoA epimerase, participate in the mechanism by which the *cis*-olefinic bonds at even and odd numbered carbon atoms of polyunsaturated fatty acids are transformed into inter-

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mediates of the β-oxidation sequence of normal saturated fatty acids[1,14,15].

Furthermore our studies with [^{3}H;^{14}C]linoleic (18:2^{9,12}) and [^{3}H;^{14}C]arachidonic acid (20:4 ^{5,8,11,14}) indicated, that these acids are either incorporated unchanged into complex phospholipids retaining the original isotope ratio or completely degraded to acetyl-CoA and further to CO_2. A partial degradation and exchange of one or two C_2-units of these two most abundant polyunsaturated fatty acids could not be observed by this sensitive isotope method[16].

As long as 30 years ago NUNN and SMEDLEY-MCLEAN[17] who studied the metabolism of docosahexaenoic acid (22:6^{4,7,10,13,16,19}) in fat deficient rats observed, that the level of eicosapentaenoic acid (20:5^{5,8,11,14,17}) in the ester lipids increased. VERDINO *et al.*[18] in analogy found that docosapentaenoic acid (22:5^{4,7,10,13,16}) is transformed by a combined chain shortening and hydrogenation into arachidonic acid (20:4^{5,8,11,14}). SCHLENK *et al.*[19,20] and KUNAU[21] confirmed these results by using labelled 22:5^{4,7,10,13,16} in their feeding experiments. SPRECHER[22] observed an increase of 20:4^{4,7,10,13} when 22:4^{6,9,12,15} was fed to fat deficient rats and of 20:4^{5,8,11,14} and of 20:3^{8,11,14} when 22:3^{10,13,16} was administered.

In the present paper the results of studies are reported which were carried out in order to gain an insight into the following systems: a) the metabolic fate of double labelled linoleic, arachidonic, eicosatrienoic (20:3^{8,11,14}), docosatrienoic (22:3 ^{10,13,16}) and docosatetraenoic acid (22:4^{7,10,13,16}), which were studied in comparative experiments *in vivo*; b) the compartment of the liver cell, in which the chain shortening occurs, was to be determined. The answer to this question would promt experiments, providing an answer as to whether: c) the partial degradation of the docosapolyenoic acids is due to a *reversal of the chain elongation reactions* or due to a *partial β-oxidation.* This should be ascertained from experiments *in vitro.*

Results

1. Chemical synthesis of substrates

In order to determine whether the polyenoic fatty acid under investigation has been degraded by a C_2-unit, either in the studies *in vivo* or *in vitro*, we labelled the substrates at carbon atom 3 with ^{14}C and, if desirable, with ^{14}C at position 3 and ^{3}H at the double bonds by partial stereospecific hydrogenation of the corresponding acetylenic precursors with ^{3}H (ref. [1,23,24]). The 3-^{14}C labelled fatty acids were synthesized in the following way: the shorter bis-homologous [1-^{14}C]polyenoic acid was synthesized according to procedures established in this laboratory. The methyl ester was reduced to the corresponding polyen-1-ol with $LiAlH_4$, the alcohol tosylated, transformed into the iodide and condensed with diethyl malonate in butanol[25,26]. Decarboxylation yielded the 3-^{14}C labelled product. The following substrates were synthesized according to this general procedure: [3-^{14}C]linoleic acid from [1-^{14}]hexadec-7,10-dienoic acid, [3-^{14}C]-eicosa-11,14-dienoic acid from [1-^{14}C]linoleic acid, [3-^{14}C]eicosa-8,11,14-trienoic acid from [1-^{14}]γ-linolenic acid and [3-^{14}C]docosa-10,13,16-trienoic and [3-^{14}C]docosa-7,10,13,16-tetraenoic acid from [1-^{14}C]eicosa-8,11,14-trienoic acid and [1-^{14}C]arachidonic acid respectively.

2. Studies in vivo with [3-^{14}C]18:2^{9,12}, [3-^{14}C]20:2 ^{11,14}, [3-^{14}C]20:3^{8,11,14}, [$^3H_8$]20:4^{5,8,11,14}, [3-^{14}C]-22:3^{10,13,16} and [3-^{14}C; $^3H_8$]22:4^{7,10,13,16}

We included in these comparative studies *in vivo* the two substrates of our former experiments, namely 18:2^{9,12} and 20:4^{5,8,11,14} and extended the substrates to the 20:3^{8,11,14} and 22:3^{10,13,16} and 20:4^{7,10,13,16} acids. All these polyenoic fatty acids belong to the linoleic acid family. In the experi-

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ments *in vivo* the ammonium salt of the polyenoic acid was injected intravenously into adult rats and the livers of the experimental animals were isolated after 6 to 8 h. The total lipid extracts of the livers from the experiments with $18:2^{9,12}$, $20:2^{11,14}$, $20:3^{8,11,14}$ and $20:4^{5,8,11,14}$ were immediately saponified, the fatty acids separated and their radioactivity determined by radio gas chromatography. If chain shortening of the 3-^{14}C labelled C_{18}- and C_{20}-polyenoic fatty acids by one C_2-unit were to occur $18:2^{9,12}$ would change to [1-^{14}C]-$16:2^{7,10}$, $20:2^{11,14}$ to $18:2^{9,12}$, $20:3^{8,11,14}$ to $18:3^{6,9,12}$ and $20:4^{5,8,11,14}$ to $18:4^{3,6,9,12}$ or $18:3^{6,9,12}$ after hydrogenation of the β, γ-double bond.

The results of these experiments however revealed that the substrate fatty acids remained either unchanged, were elongated and incorporated into the complex lipids or completely degraded as measured by the respiratory $^{14}CO_2$. Parts of the $18:2^{9,12}$, $20:2^{11,14}$ and $20:3^{8,11,14}$ acids were transformed into arachidonic acid. The sensitive isotope method confirmed and extended our previous results, which showed that no partial degradation of C_{18}- and C_{20}-polyenoic acids by chain shortening was detectable. On the other hand considerable chain shortening of $22:3^{10,13,16}$ and $22:4^{7,10,13,16}$ was observed, Table 1. After these screening experiments the livers of the experimental animals, to which the C_{22}-polyenoic acids had been administered, were homogenized and fractionated into the mitochondrial, microsomal and supernatant fractions by established methods[27]. The lipid extracts of these fractions were separated on silicic acid columns, as described previously[28], into three fractions namely the neutral lipid fraction, which also contained the free fatty acids, the phosphatidyl ethanolamine and phosphatidylcholine fractions. These fractions were transesterified and the methyl esters separated on silver nitrate impregnated silica gel H layers (solvent system: heptane/ethyl acetate 7:3) according to the number of double bonds, Table 2.

These tables clearly indicate, that the substrate fatty acids and shortened fatty acids were randomly distributed in the complex lipids of each cell fraction and in the cell fractions themselves and that no compartimentalization of the chain shortening process could be observed. The very

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Table 1. Distribution of radioactivity in fatty acids of total lipid extract after intravenous application of 3-^{14}C labelled $18:2^{9,12}$, $20:2^{11,14}$, $20:3^{8,11,14}$, $20:4^{5,8,11,14}$, $22:3^{10,13,16}$ and $22:4^{7,10,13,16}$ acids.

Fatty acid administered	% of total radioactivity in							
	16:2	18:2	18:3	20:2	20:3	20:4	22:3	22:4
18:2	—	100	—	—	—	—	—	—
20:2	—	0.1	—	26.4	20.5	40.9	—	—
20:3	—	—	0.1	—	29.1	47.9	—	—
20:4	—	—	—	—	—	100	—	—
22:3	—	—	—	—	18.7	54.3		15.4
22:4	—	—	—	—	—	44	—	56

even distrubition into phosphatidylcholine and phosphatidylethanolamine of mitochondria and microsomes become apparent in the following experiment in which the [3-^{14}C; $^{3}H_8$]$22:4^{7,10,13,16}$ acid was administered together with [1-^{14}C]16:0 acid. Phosphatidylcholines and phosphatidylethanolamines of mitochondria and microsomes were hydrolyzed with phosphilipase C from *B.cereus* and the corresponding diacylglycerides separated, according to the number of the double bonds, on silver nitrate impregnated silica gel-H plates. The tetraene fraction was isolated and the ratios of 16:0/20:4 and 16:0/(20:4 + 22:4) and 20:4/22:4 determined, Table 3.

These studies *in vivo* were then followed by those involving different cell fractions of rat liver. The localization of the partial degradation of the C_{22}-polyenoic fatty acids in one of the cell compartments should indicate whether the retroconversion of the C_{22}-acid to the C_{20}-polyenoic homologue is a reversal of the chain elongation process and which one would expect to occur in the microsomal fraction or by a single passage through the β-oxidation sequence with the loss of one acetyl-CoA. These reactions should occur in the mitochondrial fraction. Therefore we studied mainly the microsomal ($105000 \times g$ sediment) and the mitochondrial fraction ($10000 \times g$ sediment)[27]. Both fractions are known to contain the acyl-CoA synthetase for long chain fatty acids. The ammonium salts of the polyenoic acids, coenzyme A and ATP were used together with the cofactors given in the legend of Table 4.

The conversion rate by the mitochondrial fraction remained unchanged after purification by density

Table 2. Distribution of radioactivity in fatty acids of lipid classes isolated from the different subcellular fractions after intravenous administration of [3-^{14}C]22:3^{10,13,16} acid.

Fractions*	Total radioactivity [dpm]	% of extracted radioactivity	% of radioactivity in 20:3	20:4	22:3+22:4
Mitochondria					
a) *Inner membrane*	531 000	20.8			
neutral lipids					
(FFA, MG, DG, TG)			31	22	47
PC			33	67	0.1
PE			44	46	10
b) *Matrix*	4 700	0.2			
PC			45	39	16
c) *Outer membranes*	4 800	0.2			
PC			34	42	24
Microsomes	418 000	16.9			
neutral lipids					
FFA			87	10	3
TG			42	17	41
DG			65	7	28
PC			58	29	13
PE			44	33	23
105 000×g supernatant	184 200	8.4			
neutral lipids					
FFA			14	29	57
TG			31.5	8.5	60
DG			30	6	64
MG			14	24	63
PC			47	21	32
PE			37	19	44
650×g sediment	1 336 000	53.9			

* FFA = free fatty acids; MG, DG, TG = mono-, di- and triglycerides; PC = phosphatidylcholine; PE = Phosphatidylethanolamine.

Table 3. Distribution of 16:0 and 22:4 in diglycerides of phosphatidylcholines and phosphatidylethanolamines of mitochondria and microsomes after intravenous administration of [1-^{14}C]16:0 and [3-^{14}C; $^{3}H_8$] 22:4^{7,10,13,16} acids.

	Phosphatidylcholine				Phosphatidylethanolamine			
	Mitochondria		Microsomes		Mitochondria		Microsomes	
	PC	DG (tetraene fract.)	total	DG	total	DG	total	DG
16:0/20:4	1.24	0.68	1.28	0.64	1.40	0.98	1.49	1.04
16:0/(20:4 + 22:4)	0.93	0.56	1.05	0.50	0.97	0.60	0.90	0.60
20:4/22:4	4.66	4.61	4.53	3.43	2.24	1.56	1.52	1.28

Table 4. *Conversion of* [3-^{14}C]22:3^{10,13,16} *and* [3-^{14}C; ^{3}H$_8$]22:4^{7,10,13,16} to 20:3^{8,11,14} and 20:4^{5,8,11,14} acids by rat liver subcellular fractions. Each incubation mixture contained in a total volume of 2.0 ml: 0.5 μmol [3-^{14}C]22:3^{10,13,16} or [3-^{14}C; ^{3}H$_8$] 22:4^{7,10,13,16}-NH_4, 0.5 μmol CoASH, 1 μmol $NAD^{\oplus}$, 10 μmol ATP, 10 μmol $MgCl_2$, 200 μmol phosphate buffer (0.1M, pH 7.4) and 10 mg protein of each fraction, and was incubated for 1 h at 37°C.

Cell fractions	% 20:3^{8,11,14} formed	% 20:4^{5,8,11,14} formed
500 × *g* supernatant	18	21
10000 × *g* supernatant	12	9
Mitochondria complete	26.3	26
Mitochondria complete + carnitin (3 μmol)	35	44
Mitochondria — ATP	3	1.4
Mitochondria — CoA	20	12
Mitochondria — $NAD^{\oplus}$	36	31
Mitochondria sonicated for 60 sec	1.8	3
Microsomes	0.5	6

gradient centrifugation (sucrose gradient between 0.5 and 2.0M), whereas the apparent conversion catalyzed by the microsomal fraction decreased below 4% after this purification step. Ultrasonication (four 15-sec intervals at 20 mc, 5 Watts) led to a considerable loss of the enzymatic activity to approximately one fourth to one third of the control.

These results proved that the chain shortening of the docosapolyenoic acid occurs only in the mitochondria. The residual enzymatic activity in the microsomal fraction must have been due to small mitochondrial contaminations because further purification of the microsomal fraction by density gradient centrifugation decreased this activity.

We then tried to localize the enzymes responsible for the retroconversion within the mitochondrial compartments and fractionated the mitochondria into outer and inner membranes and the matrix enzymes according to SOTTOCASA *et al.*[29,30] and SCHNAITMAN *et al.*[31,32]. The purity of the submitochondrial fractions was checked by means of the marker enzymes and in addition the fractions were tested for the β-oxidation enzymes enoyl-CoA hydratase (crotonase), 3-hydroxyacyl-CoA dehydrogenase and the $\Delta^{3\,cis}$-$\Delta^{2\,trans}$-enoyl-CoA isomerase.

The distributions of the activities of the marker enzymes and β-oxidation enzymes in rat liver submitochondrial fractions are summarized in Table 5. These fractions were used for the incubations *in vitro*. The substrate was now [3-^{14}C; ^{3}H]22:4 ^{7,10,13,16}-coenzyme A ester prepared enzymatically according to KORNBERG and PRICER[33]. The results are summarized in Table 6. The inner membrane-matrix fraction of the SCHNAITMAN preparation[31] was further fractionated by osmotic shock and sedimentation of the inner membrane by ultracentrifugation at 144000 × *g* for 1 h.

The results given in Table 6 clearly indicate, that the enzymatic activity responsible for the retroconversion of 22:4^{7,10,13,16}-CoA to 20:4^{5,8,11,14} (arachidonic acid) is associated with the inner mitochondrial membrane.

In subsequent experiments we studied the influence of two possible *acceptor molecules* for the product of the partial degradation, glycerophosphate and dihydroxyacetone phosphate. The results of these experiments are given in Table 7.

The radioactive products were identified by two dimensional radio thin-layer chromatography (solvent system 1. chloroform/methanol/25% aqueous methylamine 65:25:8, 2. chloroform/methanol/acetic acid/water 25:15:4:2).

The experiments *in vivo* using doubly labelled 18:2 and 20:4 gave no indication of an exchange of carboxyl terminal C_2-units since the ^{3}H/^{14}C-ratio remained completely unchanged. Docosapolyenoic fatty acids which are largely retroconverted to eicosapolyenoic acids might, however, exhibit such an exchange. We therefore carried out the partial degradation *in vitro* with [^{3}H]docosa-7,10,13,16-tetraenoic acid in the presence of [1-^{14}C]acetyl-CoA or [2-^{14}C]*malonyl-CoA and the mitochondrial* fraction under the general conditions of the incubation. If an exchange of C-1 and C-2 was to occur the 22:4 should be doubly labelled. Neither in the presence of acetyl- nor malonyl-CoA ^{14}C-radioactivity was found in the C_{22}- or C_{20}-tetraenoic fatty acids.

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Table 5. Distribution of activities of marker enzymes in rat liver submitochondrial fractions. Specific activities are given in μmol substrate converted per min.

Fraction	Δ^{3cis}-Δ^{3trans}-Enoyl-CoA isomerase		Enoyl-CoA hydratase + 3-hydroxyacyl-CoA dehydrogenase		Malate dehydrogenase		Cytochrom *c* oxidase		Monoamine oxidase	
	[spec. act.]	[%]	[spec. act.]	[%]	[spec. act.]	[%]	[spec. act.]	[%]	[spec. act.]	[%]
Whole mitochondria	0.28	100	1.09	100	1.58	100	4.5	100	0.017	100
Inner membrane/matrix	0.48	174	2.10	193	2.55	162	9.3	211	0.017	100
Inner membrane	0.68	245	2.00	183	3.27	207	6.3	143	0.013	76
Outer membrane	0.10	36	0.59	54	0.16	10	1.4	32	0.29	1663
Matrix	0.02	7	0.36	33	0.60	38	0	0	0	0

Table 6. Retroconversion of labelled $22:4^{7,10,13,16}$-coenzyme A ester by rat liver submitochondrial fractions. Each incubation contained in a total volume of 7.5 ml: 0.1 μmol [3-^{14}C; $^{3}H_8$]$22:4^{7,10,13,16}$-CoA, 0.1 μmol $NAD^{\oplus}$, 1 mg bovine serum albumin, 250 μmol phosphate buffer pH 7.4, 2 mg protein, 0.5 μmol ATP and 0.5 μmol $Mg^{2\oplus}$ as indicated. Incubated for 90 min at 37°C.

Protein fraction	Arachidonic acid formed			
	+ ATP/$Mg^{2\oplus}$		− ATP/$Mg^{2\oplus}$	
	[nmol]	[%]	[nmol]	[%]
Whole mitochondria	51	100	45.4	100
Outer membrane	6.9	13.5	5.4	11.9
Inner membrane + matrix	55.1	108	54.0	119
Inner membrane	57.8	113	60.1	132
Matrix	4.1	8	—	—

Table 7. Partial degradation of C_{22}-polyenoic acid in the presence of dihydroxyacetone phosphate and glycero-3-phosphate.

Acceptor*	ATP/$Mg^{2\oplus}$	Radioactive product	% of partial degradation
DAP	+	PA	41.1
DAP	−	FFA	45.7
3-GP	+	PA	43.1
3-GP	−	FFA	39.5
—	+	FFA	33.2
—	−	FFA	39.4

* PA = phosphatidic acid, DAP = dihydroxy acetone phosphate, 3-GP = glycerol-3-phosphate, FFA = free fatty acid. Conditions of the incubations were the same as given under Table 6 except when 1 μmol DAP or 1 μmol 3-GP were added.

Experimental

^{3}H and ^{14}C labelled $18:2^{9,12}$, $20:2^{11,14}$, $20:3^{8,11,14}$, $20:4^{5,8,11,14}$, $22:3^{10,13,16}$ and $22:4^{7,10,13,16}$ acids were synthesized in this laboratory according to procedures described previously[23,24].

The coenzyme A ester of $22:4^{7,10,13,16}$ was prepared enzymatically according to KORNBERG and PRICER[34], crotonyl-CoA chemically according to SIMON and SHEMIN[35] and Δ^{3cis}-dodecenoyl-CoA by a modification of the method of GOLDMAN and VAGELOS[36].

[34] A. KORNBERG and W. E. PRICER, jr., J. biol. Chemistry **204**, 345 [1953].

[35] E. J. SIMON and D. SHEMIN, J. Amer. chem. Soc. **75**, 2520 [1953].

[36] P. GOLDMAN and P. R. VAGELOS, J. biol. Chemistry **236**, 2620 [1961].

For the experiments *in vivo* the fatty acids were administered intravenously as NH_4-salts.

Mitochondria from rat liver were prepared by homogenization of the minced defatted liver with a POTTER-ELVEJHEM homogenizer in 0.25M sucrose solution. Nuclei and cell debris were spun down at $650\times g$ and the mitochondria at $9500\times g$ for 10 min. The mitochondria were washed twice with isotonic sucrose. The microsomal fraction was obtained by centrifugation at $105000\times g$ for 1 h. The subfractions of mitochondria were prepared according to SOTTOCASA *et al.*[29,30] and SCHNAITMAN *et al.* [31,32]. The marker enzymes were determined by the following methods: malate dehydrogenase according to RODYN *et al.*[37], monoamine oxidase according to TABOR *et al.*[38], and cytochrome *c* oxidase according to COOPERSTEIN and LAZEROW[39]. Enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and $\Delta^{3\,cis}$-$\Delta^{2\,trans}$-enoyl-CoA isomerase were determined in the combined optical test as described before[15].

The inner membrane-matrix fraction of the SCHNAITMAN preparation was further fractionated by storing at 2°C in 0.01M phosphate buffer pH 7.4 for 1 h and sedimentation of the inner membrane at $144000\times g$ for 1 h.

Gas chromatographic analyses were carried out by a standard procedure on 200-cm columns with 15% ethylene glycol succinate polyester on kieselguhr. Radioactive components were trapped in 10 ml scintillator solution [2,5-diphenyloxazol/1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene] in toluene and counted in a liquid scintillation spectrometer Packard Model 3214. For thin-layer chromatography 0.3- to 1.0-mm silica gel H layers were used. For separation according to the degree of unsaturation argentation thin-layer chromatography using silica gel H impregnated with 20% silver nitrate was applied.

The components were visualized either with 0.02% 2,7-dichlorofluorescein in ethanol under UV-light, by charring with 50% sodium bichromate/sulfuric acid at 180°C or with ZINZADZE reagent for phospholipids. Thin-layer plates with radioactive components were scanned with either the Packard scanner model 7201 or the Berthold scanner II.

Protein was determined with the LOWRY method[40], and phosphorus according to BARTLETT[41] and modified[28].

[37] D. B. RODYN, J. W. SUTTIE and T. S. WORK, Biochem. J. **83**, 29 [1962].

[38] C. W. TABOR, H. TABOR and S. M. ROSENTHAL, Methods in Enzymol. **2**, 390 [1955].

[39] S. J. COOPERSTEIN and A. LAZEROW, J. biol. Chemistry **189**, 665 [1951].

[40] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chemistry **193**, 265 [1951].

[41] G. R. BARTLETT, J. biol. Chemistry **234**, 466 [1959].

Discussion

Previous experiments in this laboratory with C_{18}- and C_{20}-polyenoic fatty acids labelled specifically at carbon atom 3 with ^{14}C and ^{3}H in the hydrocarbon chain had demonstrated that these acids retained their isotope ratio so far as the sensitivity of the method used could ascertain and furthermore that no homologous acids shortened by two carbon atoms could be isolated. These results were confirmed in these studies with $18:2^{9,12}$ and $20:4^{5,8,11,14}$ and extended to $20:2^{11,14}$ and $20:3^{8,11,14}$ acids. These acids are either degraded completely, retain their structure or are desaturated to arachidonic acid and are incorporated into the ester lipids.

As indicated in the introduction the chain shortening of C_{22}-polyenoic acids has been observed over many years. All these observations were made in feeding experiments mainly with $22:6^{4,7,10,13,16,19}$ and $22:5^{7,10,13,16,19}$ acids. Our studies *in vivo* reported in the first part of this paper, in which specifically labelled $22:3^{10,13,16}$ and $22:4^{7,10,13,16}$ acids were used as substrates, confirm these data. Two mechanisms for the retroconversion of the C_{22}- to the C_{20}-polyenoic acids could be envisaged from theoretical reasoning:

1. The release of a C_2-unit from the carboxyl end of the C_{22}-polyenoic acid could be due to a reversal of the chain elongation sequence. These reactions are confined predominantly to the enzyme system located on the endoplasmic reticulum.

2. The chain shortening could be the result of one passage of the C_{22}-polyenoic acid through the β-oxidation sequence. This process should take place *in the mitochondrion.*

We first hoped to decide from the distribution of the C_{22}-substrate polyenoic acid and its corresponding degradation product, the C_{20}-polyenoic acid, in which compartment of the cell the participating enzymes reside. However, no particular distribution of the C_{20}-acids in any of the subcellular fractions could be observed. A rapid exchange of the labelled fatty acids, incorporated mainly into the phospholipids, throughout all subcellular compartments had taken place. Our observation is in agreement with a recent report by WIRTZ and ZILVERSMIT[42].

[42] K. W. A. WIRTZ and D. B. ZILVERSMIT, J. biol. Chemistry **243**, 3596 [1968].

Our studies *in vivo* were then extended to experiments *in vitro*. We observed that the microsomal fraction of rat liver showed no enzymic activity leading to a chain shortening of either $22:3^{10,13,16}$ or $22:4^{7,10,13,16}$ acids. Therefore the reverse of the chain elongation process could be excluded.

However, since the whole mitochondrial fraction was able to degrade the C_{22}-substrates to their C_{20}-homologues we further subfractionated the mitochondria in order to prove weather a partial β-oxidation does indeed occur with a high chain length specificity. The procedure of SOTTOCASA *et al.*[29,30] and of SCHNAITMAN *et al*[31,32] were applied to the subfractionation of the mitochondria in outer and inner membrane and matrix. The enzyme(s) involved in the chain length specific reactions of this partial degradation is (are) sensitive to even short interval ultrasonication. ATP-$Mg^{2\oplus}$ has a protective effect. Also freezing and thawing leads to the complete loss of enzymic activity. The SOTTOCASA procedure involves a sonication step. Therefore we preferred the method of SCHNAITMAN which in our hands also yielded preparations with much higher uniform activities of the marker enzymes in the matrix, inner and outer membrane fractions of the mitochondrion. We further separated the inner membrane-matrix fraction by rupture of the inner membrane in a hyposmotic solution (0.01M phosphate buffer pH 7.4) and centrifugation of the inner membrane. However the malate dehydrogenase activity still partly associated with the inner membrane may be due to an incomplete separation of the "matrix protein" from the inner membrane. The incubations of $22:4^{7,10,13,16}$ and these mitochondrial subfractions clearly showed that the enzymes, which catalyze the retroconversion are not only associated with the inner membrane-matrix fraction of the SCHNAITMAN preparation but with the inner membrane itself. They are furthermore closely associated with the enzyme activities of the β-oxidation which we tested here as marker enzymes, namely enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and $\Delta^{3\,cis}$-$\Delta^{2\,trans}$-enoyl-CoA isomerase. The chain shortening process is dependent on $NAD^{\oplus}$. Carnitine, which is required for the passage of the mitochondrial membrane can be omitted when the inner mitochondrial membrane fraction is used as enzyme source and the coenzyme A ester as substrate. This experimental evidence indicates that the breakdown of the C_{22}- to their respective C_{20}-polyenoic acids proceeds at the site of and *via* the β-oxidation enzymes. The mechanism suggested by VERDINO *et al.*[18] who postulated a reaction involving H_2O_2 can be excluded. In addition there must be an enzyme or a regulatory mechanism which pulls off the C_{20}-intermediate from the multienzyme complex of the β-oxidation. The final answer to this question cannot be offered at this moment. We tested the possibility that an acceptor molecule for the C_{20}-fatty acid might act as a regulatory factor. Dihydroxyacetone phosphate[43] and glycerol 3-phosphate[33] are acceptor molecules in the biosynthesis of complex lipids. When these compounds are present in the *in vitro* system, no $22:4^{7,10,13,16}$ and $20:4^{5,8,11,14}$ acids can be isolated as free fatty acids but both are bound in phosphatidic acid in the same ratio as when they are formed without these acceptors. There is neither a preference for the reaction product in the acylation reaction nor an increase in the chain shortening as compared to the control experiments. Therefore the regulation in this partial β-oxidation is not *via* an acyl transferase for the C_{20}-polyenoic fatty acids. In our experiments *in vitro* we were able to carry out the partial degradation of the coenzyme A esters of C_{22}-polyenoic acids to their respective C_{20}-homologues in a yield equal to or better than those observed in the whole animal experiments (60—70%), when the inner membrane or inner membrane-matrix of the mitochondria was used as the enzyme source. We also studied the possibility of a measurable exchange of carbon atoms 1 and 2 of docosa-7,10,13,16-tetraenoic acid and arachidonic acid in the mitochondria. The two ^3H-labelled acids were incubated with whole mitochondria in the presence of either [1-^{14}C]acetyl-CoA or [2-^{14}C]malonyl-CoA, ATP, CoA, NADH and carnitine. An exchange would lead to a doubly labelled reaction product. However no extension of arachidonic acid to docosatetraenoic acid and no exchange of the terminal two C atoms of docosatetraenoic acid occurred whereas the retroconversion of 22:4 to 20:4 was catalyzed with a yield of between 30 and 50%. These results suggest that the partial degradation in the mitochondria proceeds only in one direction. Since our studies revealed that the partial degradation of the docosapolyenoic acids to eicosapolyenoic acids occurs only in the mitochondria, and furthermore a rapid exchange of

[43] A. K. HAJRA and B. W. AGRANOFF, J. biol. Chemistry **243**, 1617 [1968].

the fatty acids and lipids between the intra- and extramitochondrial compartment became evident from our studies *in vivo*, a chain elongation of the eicosapolyenoic acid derived from the docosapolyenoic acid may be catalyzed by the microsomal malonyl-CoA dependent chain elongation system. The analogous reaction has been demonstrated previously with an adrenal microsomal preparation[3].

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