Remodeling of Phospholipid Fatty Acids in Mitochondrial Membranes of Estivating Snails

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ABSTRACT: The effects of estivation on the phospholipid-specific fatty acid composition of mitochondrial membranes in the hepatopancreas of the terrestrial snail *Cepaea nemoralis* were investigated. The fatty acid composition of all phospholipids was significantly altered in snails estivating for 6 wk, indicating that substantial remodeling occurs. The most profound changes occurred in cardiolipin (CL). CL of estivating snails was 13-fold more saturated, contained 9-fold more monoenes, and had 45% fewer polyenes than in active snails. These differences were due, in part, to a reduction in linoleic acid (18:2n-6) content of CL from estivators. As in mammals, CL of active snails appears to preferentially incorporate 18:2n-6, which accounts for 60% of the acyl chains in this phospholipid. This proportion was reduced by 50% in estivators. Changes in the fatty acyl content of other phospholipids of estivating snails included increased monoenes in phosphatidylethanolamine (PE) and phosphatidylinositol, reduced ratios of n-3/n-6 polyenes in PE and phosphatidylcholine (PC), and an increased n-3/n-6 ratio in phosphatidylserine (PS). Arachidonic acid (20:4n-6) levels were reduced in PS but increased in CL and PC. Taken together, these alterations to fatty acid composition are consistent with decreased biological activity of membrane-related processes which occur in conjunction with the reduction of mitochondrial aerobic metabolism observed during estivation.

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Depression of basal metabolic rate is a common physiological strategy, allowing organisms to prolong the duration of their tolerance of suboptimal environmental conditions (1,2). Profound metabolic rate depression occurs during hibernation, torpor, and estivation. Suppression of mitochondrial respiration is a key component of strategies to reduce basal metabolic rate. Indeed, in hibernating ground squirrels, electron transfer is inhibited by 70 to 80% at the site of ubiquinol/cytochrome c_1, and adjustments of mitochondrial membrane composition are implicated in the mediation of this reduction (3). Changes to mitochondrial membrane composition have been studied in mammalian hibernators (4). However, hibernation and torpor are characterized by reductions in both metabolic rate and body temperature, and thus it is difficult to attribute changes of membrane phospholipid composition to either of these parameters. Several authors have attempted to overcome the confounding effects of temperature and metabolic rate reduction by comparing membrane compositional changes in active and quiescent overwintering animals (5,6), thus controlling for responses induced by lowered temperature. Nonetheless, interspecies differences still remain. Estivation, however, provides a superior model for studies of this nature. It typically occurs during environmental drought as a means to avoid desiccation (1). Metabolic rate reduction occurs in the absence of temperature change or significant cellular water loss. The extent of metabolic depression in estivators is at least as great as that observed during hibernation and torpor. Estivating terrestrial snails, for example, typically undergo a metabolic rate reduction of 85% (2).

As the functional mileu of the electron transport chain and many of the transporters and channels associated with oxidative phosphorylation, the mitochondrial membranes represent a potentially powerful site for the regulation of metabolism. Mitochondrial membranes are composed principally of proteins and phospholipids, and the function of many of the proteins associated with the membranes is responsive to, and in many cases dependent upon, surrounding phospholipids (7,8). Altering the composition of associated phospholipids can effect large changes in protein function (7). Modifications to the phospholipid environment include substitution of one phospholipid species for another, and/or modification of the acyl chains of particular phospholipids. Previously, we demonstrated that changes in the absolute and proportional amounts of individual phospholipids were altered in estivating snails. Here, we present results which indicate that a phospholipid-specific fatty acid remodeling occurs in mitochondrial membranes which is consistent with reduced rates of metabolic functions during estivation.

MATERIALS AND METHODS

Experimental animals. Several *Cepaea nemoralis* were collected in early summer, kept in terraria in the laboratory for *ca.* 6 wk, and fed a diet of "iceberg" lettuce. A group of these snails was removed to a dry terrarium, and food was withheld to induce estivation which lasted for 6 wk.

Mitochondrial isolation. Hepatopancreas from active or estivating snails was excised and immersed in 10 vol of mitochondrial isolation buffer (100 mM sucrose, 20 mM Hepes),

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Abbreviations: CL, cardiolipin; H_{II}, inverse hexagonal phase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

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0.5% bovine serum albumin (BSA), pH 7.5), and homogenized by three passes with a Potter-Elvehjem homogenizer (Fisher Scientific Ltd., Whitby, Ontario, Canada). Homogenates were centrifuged at $150 \times g$ for 10 min and the pellet discarded. The remaining supernatant was centrifuged at $5000 \times g$ for 10 min, and the pellet "washed" by repeating this step twice. This procedure consistently gave a recovery of *ca.* 85% of mitochondria (9).

Purity of the mitochondrial preparation and recovery of mitochondria did not differ significantly ($P > 0.05$) between active and estivating snails based on the distribution of cytochrome c oxidase (mitochondrial marker), peroxidase (peroxisomal marker), and the proportional content of the phospholipid sphingomyelin (used to mark nonmitochondrial membranes, including nuclear membrane, endoplasmic reticulum, and lysosomal and plasma membranes). Recoveries of total cytochrome oxidase activities in the "mitochondrial fraction" of active and estivating snails were 82.4 ± 5.1 and 88.8 ± 4.0% ($n = 4$), respectively. Only 3.5 ± 1.0% of total peroxidase activity was recovered in the mitochondrial fraction of active snails, and 3.8 ± 0.8% ($n = 4$) in estivating snails. The proportional content of sphingomyelin in the mitochondrial fraction was 2.4 ± 1.5% ($n = 7$) in active snails and 4.2 ± 1.5% ($n = 8$) in estivators.

Total mitochondrial lipids were extracted by the method of Bligh and Dyer (10). Phospholipids were separated from neutral lipids and each other by thin-layer chromatography and methylated, as in Holub and Skeaff (11). Bands were visualized by using dichlorofluorescein. There were no band overlaps. An internal standard (17:0) was added to the phospholipid fatty acids prior to methylation. Individual fatty acids from each phospholipid fraction were separated and identified by gas chromatography using a reversed-phase DB-225 fused silica column (J&W Scientific, Folsom, CA) as described by Glémet and Ballantyne (12).

A small sample (1 g) of the diet (lettuce) was homogenized in 50 mM imidazole buffer using three 10-s bursts with a Polytron PT10 unit (Kinematica GmbH, Luzern, Switzerland). Lipid extraction of the homogenate was as above. Individual fatty acids were separated and quantified as above.

Chemicals. Chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Ltd. and were of the highest purity available. The fatty acid standard was obtained from Nu-Chek Prep Inc. (Elysian, MN) and was augmented by the addition of a menhaden extract.

Statistical analysis. Proportional phospholipid fatty acid compositional data were arcsine transformed where necessary to normalize the data and were compared by using Student's *t*-tests. *P*-values were adjusted (based on the number of tests made and degrees of freedom) to compensate for the use of multiple *t*-tests in comparing the fatty acid compositions of each phospholipid from active and estivating snails (13).

RESULTS

The snail diet (lettuce) was composed primarily of three fatty acids, 16:0, 18:2n-6 and 18:3n-3, which combine to account for 83% of total fatty acids (Table 1). 18:2n-6 was the most abundant fatty acid in lettuce, at almost 42% of total. Lettuce contained a large proportion (64%) of 18-carbon fatty acids, a lower proportion (29%) of 16-carbon fatty acids, and trace amounts of 14, 20, 22 and 24-carbon fatty acids.

Phospholipids from hepatopancreas mitochondria of active snails contained lower proportions (47%) of 18-carbon fatty acids than dietary lettuce, and greater proportions of their elongation products, especially 20-carbon fatty acids (37%). Some of the most common fatty acids in hepatopancreas mitochondrial phospholipids of active snails are 18:2n-6 (18%), 18:3n-3 (8%), and 20:4n-6 (18%) (Table 2).

The fatty acid composition of mitochondrial phospholipids was altered dramatically in estivating snails (Table 2). The proportion of 18:2n-6, 18:3n-3, and 18:4n-3 decreased by 60,

TABLE 1
Percentages of Individual Fatty Acids in Lettuce (average of two determinations)[a]

Fatty acid	Amount (mol %)
Nonessential	
14:0	0.82
14:1	0.12
16:0	25.04
16:1	3.65
18:0	1.81
18:1	1.92
20:0	0.40
20:1	0.41
22:0	0.88
22:1	0.93
24:0	1.27
24:1	0.73
n-3 Polyunsaturated	
18:3n-3	15.92
18:4n-3	2.69
20:3n-3	0.24
22:5n-3	0.32
22:6n-3	0.11
n-6 Polyunsaturated	
18:2n-6	41.93
20:2n-6	0.86
20:3n-6	n.d.
20:4n-6	0.23
22:2n-6	n.d.
22:4n-6	n.d.
22:5n-6	0.48
Total	100
Total saturates	30.21
Total monoenes	7.75
Total polyenes	62.76
n-3 Polyenes	19.26
n-6 Polyenes	43.50
n-3/n-6	0.44
Mononenes/polyenes	0.12
Unsaturation index	157.33
Chain length	17.00

[a]n.d., Not detectable.

TABLE 2
Percentages of Individual Fatty Acids in Cardiolipin of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a]

Fatty acid	Active (n = 7)	Estivating (n = 8)
Nonessential		
14:0	0.27 ± 0.14	5.26 ± 2.35
14:1	0.49 ± 0.43	2.60 ± 0.87
16:0	0.86 ± 0.23	9.07 ± 2.46
16:1	0.78 ± 0.42	12.18 ± 3.64
18:0	0.60 ± 0.31	5.96 ± 2.41
18:1	1.32 ± 0.47	5.59 ± 2.06
20:1	0.08 ± 0.04	2.70 ± 0.65[b]
23:0	0.11 ± 0.05	3.33 ± 1.47[b]
18:3n-3	20.58 ± 0.87	8.85 ± 2.04[b]
18:4n-3	8.57 ± 1.45	4.07 ± 1.64
20:3n-3	0.63 ± 0.08	n.d.
20:5n-3	0.18 ± 0.07	1.14 ± 0.89
22:5n-3	n.d.	0.19 ± 0.19
22:6n-3	n.d.	0.34 ± 0.20
n-6 Polyunsaturated		
18:2n-6	60.19 ± 1.67	29.68 ± 5.84[b]
20:2n-6	0.85 ± 0.08	1.31 ± 0.29
20:3n-6	0.71 ± 0.13	1.25 ± 0.17
20:4n-6	3.67 ± 0.27	5.11 ± 0.98[b]
22:2n-6	n.d.	0.11 ± 0.11
Total	100	100
Total saturates	1.85 ± 0.48	24.75 ± 6.29[b]
Total monoenes	2.67 ± 0.64	23.07 ± 4.69[b]
Total polyenes	95.48 ± 1.11	52.18 ± 9.40[b]
n-3 Polyenes	29.99 ± 1.43	14.58 ± 3.98
n-6 Polyenes	65.49 ± 1.85	37.60 ± 6.34[b]
n-3/n-6	0.46 ± 0.03	0.37 ± 0.09
Monoenes/polyenes	0.03 ± 0.01	0.81 ± 0.31
Unsaturation index[c]	240.76 ± 2.91	161.64 ± 22.52[b]
Chain length[d]	17.23 ± 0.04	17.16 ± 0.20

[a]Values are presented as means ± SE; n.d. = not detectable.
[b]Significantly different from active values, $\alpha = 0.0034$.
[c]Unsaturation index = $\sum m_i \cdot n_i$; where m_i is the mole percentage and n_i is the number of C–C double bonds in fatty acid "i".
[d]Mean chain length = $\sum f_i \cdot c_i$; where f_i is the mole fraction and c_i is the number of carbon atoms in fatty acid "i".

61, and 63%, respectively, in hepatopancreas mitochondria of estivating snails. 18-carbon fatty acids constituted only 30% of total fatty acids in estivators, compared with 47% in active snails (see above). These changes were offset by greater proportions of shorter chain fatty acids (14- and 16-carbons) and longer chain (22-carbons) fatty acids, which increased from 10 to 18% and from 5 to 9%, respectively. The phospholipid fatty acids of estivating snails contained significantly more monoenes and fewer polyenes. The reduced polyene content of mitochondrial membranes from estivators resulted primarily from a lower content of n-3 fatty acids, the proportional occurrence of which was reduced by 51%.

The greatest differences between the fatty acid composition of active and estivating snail mitochondria occur in the major constituent phospholipids of the mitochondrial membranes [phosphatidylcholine ((PC), phosphatidylethanolamine (PE) and cardiolipin (CL), which constitute 37.7, 35.2, and 11.4% of total phospholipids in mitochondria from the hepatopancreas of active snails (14)]. Perhaps the most profound alterations of fatty acid composition occurred in CL (Table 3). A 50% reduction in 18:2n-6 content was observed in CL which, in combination with a 57% decrease in 18:3n-3, accounted for a 55% decrease in the total polyene content of CL from estivating, compared to active, snails. While polyene levels decreased, saturated fatty acids increased 13-fold, and monoenes increased 9-fold. This resulted in a 33% reduction in CL unsaturation index.

Unsaturation index was not altered during estivation in any other phospholipid species. However, in PE, the proportion of monoenes increased 48%, due in part to an almost fourfold increase in 16:1 content (Table 4). Membranes of estivators contained 43% fewer n-3 polyenes, largely due to significant reductions in 18-carbon n-3 fatty acids. This resulted in a lowering, by 43%, of the n-3/n-6 ratio in estivating snail mitochondria.

TABLE 3
Percentages of Individual Fatty Acids in Phosphatidylethanolamine of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active (n = 7)	Estivating (n = 8)
Nonessential		
14:0	1.74 ± 0.76	2.37 ± 0.71
14:1	1.30 ± 0.36	2.00 ± 0.64
16:0	2.56 ± 0.20	4.70 ± 0.71
16:1	1.67 ± 0.34	6.25 ± 0.84[b]
18:0	15.28 ± 0.90	9.36 ± 0.58[b]
18:1	4.76 ± 0.24	3.84 ± 0.62
20:0	0.45 ± 0.11	0.57 ± 0.09
20:1	3.26 ± 0.15	4.20 ± 0.23
23:0	0.82 ± 0.09	1.37 ± 0.16[b]
n-3 Polyunsaturated		
18:3n-3	4.06 ± 0.22	1.24 ± 0.23[b]
18:4n-3	1.26 ± 0.19	0.43 ± 0.07[b]
20:3n-3	3.42 ± 0.63	1.10 ± 0.30
20:4n-3	0.08 ± 0.08	0.74 ± 0.27
20:5n-3	2.34 ± 0.28	2.20 ± 0.42
22:5n-3	0.31 ± 0.08	0.29 ± 0.13
22:6n-3	0.45 ± 0.02	0.85 ± 0.14
n-6 Polyunsaturated		
18:2n-6	10.41 ± 0.79	5.10 ± 0.40[b]
20:2n-6	7.91 ± 0.17	10.14 ± 0.58
20:3n-6	3.60 ± 1.21	5.68 ± 0.77
20:4n-6	27.59 ± 1.87	28.49 ± 1.71
22:4n-6	2.12 ± 0.07	3.09 ± 0.18[b]
22:5n-6	4.58 ± 0.29	5.99 ± 0.17
Total	100	100
Total saturates	20.85 ± 1.62	18.37 ± 1.45
Total monoenes	10.99 ± 0.21	16.29 ± 1.51[b]
Total polyenes	68.16 ± 1.49	65.34 ± 2.71
n-3 Polyenes	11.93 ± 0.87	6.84 ± 0.49[b]
n-6 Polyenes	56.22 ± 1.26	58.50 ± 2.33
n-3/n-6	0.21 ± 0.02	0.12 ± 0.01[b]
Monoenes/polyenes	0.16 ± 0.01	0.26 ± 0.04[b]
Unsaturation index[c]	243.85 ± 6.90	247.84 ± 8.22
Chain length[d]	18.91 ± 0.07	18.97 ± 0.11

[a–d]See Table 2 for footnotes.

TABLE 4
Percentages of Individual Fatty Acids in Phosphatidylcholine of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active (n = 7)	Estivating (n = 8)
Nonessential		
14:0	0.45 ± 0.14	1.18 ± 0.63
14:1	1.95 ± 1.25	0.98 ± 0.45
16:0	7.32 ± 0.38	6.64 ± 0.28
16:1	1.45 ± 0.56	2.57 ± 0.68
18:0	2.38 ± 0.12	4.06 ± 0.40[b]
18:1	13.93 ± 0.88	11.22 ± 1.14
20:0	0.32 ± 0.03	0.50 ± 0.10
20:1	3.37 ± 0.39	4.60 ± 0.40
23:0	0.21 ± 0.18	0.10 ± 0.05
n-3 Polyunsaturated		
18:3n-3	8.14 ± 0.44	2.37 ± 0.50[b]
18:4n-3	2.83 ± 0.47	1.00 ± 0.15[b]
20:3n-3	3.77 ± 0.44	1.70 ± 0.12[b]
20:4n-3	n.d.	0.12 ± 0.09
20:5n-3	1.35 ± 0.20	0.90 ± 0.15
22:6n-3	1.10 ± 0.16	2.35 ± 0.58
n-6 Polyunsaturated		
18:2n-6	19.69 ± 1.05	9.09 ± 0.62[b]
20:2n-6	12.62 ± 0.42	16.11 ± 0.64[b]
20:3n-6	2.53 ± 0.44	4.58 ± 0.67
20:4n-6	12.36 ± 0.59	21.01 ± 0.91[*b]
22:2n-6	n.d.	n.d.
22:4n-6	0.39 ± 0.03	0.60 ± 0.04
22:5n-6	3.65 ± 0.22	8.24 ± 0.66[b]
Total	100	100
Total saturates	10.77 ± 0.44	12.48 ± 1.13
Total monoenes	20.70 ± 1.30	19.38 ± 1.50
Total polyenes	68.53 ± 1.64	68.14 ± 2.06
n-3 Polyenes	17.28 ± 1.16	8.50 ± 0.67[b]
n-6 Polyenes	51.26 ± 1.59	59.64 ± 2.22
n-3/n-6	0.34 ± 0.03	0.15 ± 0.02[b]
Monoenes/polyenes	0.31 ± 0.03	0.29 ± 0.03
Unsaturation index[c]	222.82 ± 3.90	246.51 ± 6.74
Chain length[d]	18.33 ± 0.08	19.03 ± 0.12[b]

[a–d]See Table 2 for footnotes.

TABLE 5
Percentages of Individual Fatty Acids in Phosphatidylserine of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active (n = 7)	Estivating (n = 8)
Nonessential		
14:0	0.45 ± 0.24	3.07 ± 1.54
14:1	0.93 ± 0.45	2.56 ± 0.90
16:0	7.26 ± 0.80	7.51 ± 2.31
16:1	2.14 ± 0.61	4.92 ± 1.94
18:0	25.40 ± 1.40	20.15 ± 4.56
18:1	5.96 ± 0.50	7.77 ± 2.08
20:0	1.34 ± 0.41	2.40 ± 0.45
20:1	11.07 ± 2.33	5.55 ± 1.79
23:0	0.14 ± 0.11	0.78 ± 0.36
n-3 Polyunsaturated		
18:3n-3	9.57 ± 0.47	25.35 ± 9.71
18:4n-3	2.06 ± 0.47	1.70 ± 0.68
20:3n-3	0.57 ± 0.33	n.d.
20:5n-3	0.26 ± 0.13	n.d.
22:6n-3	0.19 ± 0.10	2.35 ± 0.58
n-6 Polyunsaturated		
18:2n-6	2.95 ± 0.55	3.03 ± 0.91
20:2n-6	2.03 ± 0.35	1.49 ± 0.70
20:3n-6	2.17 ± 0.41	1.43 ± 0.47
20:4n-6	23.87 ± 1.32	11.34 ± 1.35[b]
22:4n-6	0.16 ± 0.06	n.d.
22:5n-6	1.49 ± 0.25	0.85 ± 0.30
Total	100	100
Total saturates	34.60 ± 0.89	33.91 ± 5.58
Total monoenes	20.10 ± 1.60	20.81 ± 4.62
Total polyenes	45.30 ± 1.34	45.29 ± 9.87
n-3 Polyenes	12.64 ± 0.85	27.13 ± 9.94
n-6 Polyenes	32.67 ± 1.64	18.16 ± 2.31[b]
n-3/n-6	0.40 ± 0.04	1.84 ± 0.70
Monoenes/polyenes	0.45 ± 0.04	0.75 ± 0.20
Unsaturation index[c]	181.18 ± 3.97	167.04 ± 23.87
Chain length[d]	18.24 ± 0.09	16.93 ± 0.42

[a–d]See Table 2 for footnotes.

No statistically significant changes to proportions of saturates, monoenes, or polyenes were observed in any other phospholipids. However, PC showed a similar decrease (51%) in n-3 fatty acid content in estivators (Table 5). This occurred primarily through significant reductions to 18:3n-3, 18:4n-3, and 20:3n-3 contents during estivation. The n-3/n-6 ratio of PC from estivating snails was thus decreased by 56%.

PC was the only phospholipid to show a change in average fatty acid chain length between experimental groups. PC from mitochondria of estivating snails contained longer fatty acyl chains on average than in active snails.

The n-3/n-6 polyene ratio appeared to be 4.5-fold greater in phosphatidylserine from estivating snails (Table 6), though a relatively large standard error made this statistically insignificant at $\alpha = 0.0034$. The altered value of the n-3/n-6 ratio was largely due to a statistically significant 50% decrease in 20:4n-6 and a concomitant, but not significant, 2.6-fold increase in 18:3n-3.

In phosphatidylinositol (PI), only 20:1 and 16:1 content were altered in estivators (Table 7). Proportions of both increased, which was reflected in a doubling of the proportion of monoenes in PI, though this was also not significant at $\alpha = 0.0034$.

Arachidonic acid (20:4n-6) was present in trace amounts in lettuce (Table 1), but occurred as a major constituent fatty acid of all phospholipids, except for CL, where it accounted for less than 4% of total fatty acids in active snails. 20:4n-6 was enriched 70% in PC during estivation, while PS 20:4n-6 content decreased by 52%.

DISCUSSION

The phospholipid composition of mitochondrial membranes from *C. nemoralis* hepatopancreas is dramatically altered during estivation. Many aspects of this remodeling indicate a reduced biological activity of the mitochondrion during estivation, when metabolic rate is reduced to about 15% of normal

TABLE 6
Percentages of Individual Fatty Acids in Phosphatidylinositol of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active ($n = 7$)	Estivating ($n = 8$)
Nonessential		
14:0	0.80 ± 0.45	4.68 ± 1.65
14:1	0.62 ± 0.32	1.39 ± 0.70
16:0	24.07 ± 0.94	19.05 ± 2.36
16:1	2.61 ± 0.58	7.89 ± 1.51[b]
18:0	26.45 ± 0.89	23.67 ± 1.18
18:1	4.95 ± 1.00	7.46 ± 1.05
20:0	1.16 ± 0.10	0.93 ± 0.20
20:1	2.60 ± 0.15	5.36 ± 0.73[b]
23:0	0.20 ± 0.10	0.19 ± 0.16
n-3 Polyunsaturated		
18:3n-3	4.21 ± 0.26	2.67 ± 0.41
18:4n-3	1.12 ± 0.16	0.94 ± 0.45
20:3n-3	1.01 ± 0.26	0.51 ± 0.27
20:4n-3	n.d.	n.d.
20:5n-3	0.67 ± 0.11	0.43 ± 0.17
22:6n-3	0.09 ± 0.06	0.30 ± 0.12
n-6 Polyunsaturated		
18:2n-6	2.32 ± 0.30	1.05 ± 0.35
20:2n-6	1.63 ± 0.24	1.57 ± 0.20
20:3n-6	2.52 ± 0.49	2.48 ± 0.57
20:4n-6	22.59 ± 0.77	18.03 ± 2.68
22:4n-6	0.04 ± 0.04	0.14 ± 0.6
22:5n-6	0.32 ± 0.13	1.27 ± 0.42
Total	100	100
Total saturates	52.68 ± 1.64	48.52 ± 2.89
Total monoenes	10.78 ± 1.41	22.10 ± 2.31
Total polyenes	36.54 ± 1.58	29.38 ± 4.05
n-3 Polyenes	7.10 ± 0.37	4.85 ± 0.88
n-6 Polyenes	29.44 ± 1.31	24.54 ± 3.42
n-3/n-6	0.24 ± 0.01	0.20 ± 0.04
Monoenes/polyenes	0.30 ± 0.04	0.98 ± 0.29
Unsaturation index[c]	142.42 ± 4.74	131.04 ± 14.64
Chain length[d]	17.86 ± 0.05	17.62 ± 0.25

[a–d]See Table 2 for footnotes.

TABLE 7
Percentages of Individual Fatty Acids in Phosphatidylinositol of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active ($n = 7$)	Estivating ($n = 8$)
14:0	0.80 ± 0.45	4.68 ± 1.65
14:1	0.62 ± 0.32	1.39 ± 0.70
16:0	24.07 ± 0.94	19.05 ± 2.36
16:1	2.61 ± 0.58	7.89 ± 1.51[b]
18:0	26.45 ± 0.89	23.67 ± 1.18
18:1	4.95 ± 1.00	7.46 ± 1.05
18:2n-6	2.23 ± 0.30	1.05 ± 0.35
18:3n-3	4.21 ± 0.26	2.67 ± 0.41
18:4n-3	1.12 ± 0.16	0.94 ± 0.45
20:0	1.16 ± 0.10	0.93 ± 0.20
20:1	2.60 ± 0.15	5.36 ± 0.73[b]
20:2n-6	1.63 ± 0.24	1.57 ± 0.20
20:3n-6	2.52 ± 0.49	2.48 ± 0.57
20:4n-6	22.59 ± 0.77	18.03 ± 2.68
20:3n-3	1.01 ± 0.26	0.51 ± 0.27
20:4n-3	n.d.	n.d.
20:5n-3	0.67 ± 0.11	0.43 ± 0.17
22:0	n.d.	n.d.
22:1	n.d.	n.d.
22:2n-6	n.d.	n.d.
23:0	0.20 ± 0.10	0.19 ± 0.16
22:4n-6	0.04 ± 0.04	0.14 ± 0.6
22:5n-6	0.32 ± 0.13	1.27 ± 0.42
22:5n-3	n.d.	n.d.
22:6n-3	0.09 ± 0.06	0.30 ± 0.12
24:0	n.d.	n.d.
24:1	n.d.	n.d.
Total	100	100
Total saturates	52.68 ± 1.64	48.52 ± 2.89
Total monoenes	10.78 ± 1.41	22.10 ± 2.31
Total polyenes	36.54 ± 1.58	29.38 ± 4.05
n-3 Polyenes	7.10 ± 0.37	4.85 ± 0.88
n-6 Polyenes	29.44 ± 1.31	24.54 ± 3.42
n-3/n-6	0.24 ± 0.01	0.20 ± 0.04
Monoenes/polyenes	0.30 ± 0.04	0.98 ± 0.29
Unsaturation index[c]	142.42 ± 4.74	131.04 ± 14.64
Chain length[d]	17.86 ± 0.05	17.62 ± 0.25

[a–d]See Table 2 for footnotes.

(1,2). These changes are consistent with the inhibition of certain mitochondrial membrane-bound proteins, a stabilizing of the bilayer, and a reduced propensity toward hexagonal phase formation.

Perhaps the most dramatic changes to constituent fatty acids occurred in CL, a unique phospholipid which typically is found exclusively in the mitochondrial inner membrane (7). CL is found in close association with certain mitochondrial proteins, including the mono-, di-, and tricarboxylate carriers, and also carnitinepalmitoyl translocase, cytochrome c oxidase, ADP/ATP exchanger, and phosphate transporter (7). Many of these proteins do not function, or function submaximally, in its absence. In many cases, the ability of CL to stimulate protein function is dependent upon its fatty acyl composition (7,15,16). For example, several studies have demonstrated a specific requirement of the respiratory chain enzyme cytochrome c oxidase for CL with 18:2n-6 acyl chains. In rats fed diets deficient in 18:2n-6, which results in CL 18:2n-6 being replaced with other fatty acids (17), mitochondrial function (18), and cytochrome c oxidase activity (15) are both decreased by as much as 50%. This loss of activity has been shown to result directly from the presence of 18:2n-6-deficient CL, as activity can be fully recovered when delipidated cytochrome c oxidase is reconstituted with 18:2n-6/18:2n-6 CL (15). In estivating *Cepaea*, cytochrome c oxidase activity is reduced by 85% (14). This could be mediated in part by the significant reduction of CL 18:2n-6 content. As in mammals, *Cepaea* CL was particularly enriched in 18:2n-6, which accounted for 60% of all fatty acids in CL of active snails. The 18:2n-6 content of CL from estivating *Cepaea* decreased by 50%. A similar decrease in CL 18:2n-6 content in rats fed an 18:2n-6-deficient diet results in a 26% decrease in cytochrome c oxidase activity (15), suggesting that, during estivation, the reduced 18:2n-6 content of CL may play a role in

the observed reduction of cytochrome c oxidase activity. The activities of other CL-requiring mitochondrial membrane proteins which have specific interactions with 18:2n-6 acyl chains (7,15,16) may be similarly suppressed.

The altered fatty acyl composition of CL changes the molecular geometry of this phospholipid, and thus its functional properties in the membrane (19,20). CL of estivating snails was threefold more saturated, contained ninefold more monoenes, and had 45% fewer polyenes than that of active snails. Thus, the unsaturation index of CL from estivating snails was 33% reduced from control values. The more highly saturated CL found in estivating snails effects changes in the molecular geometry of CL which decrease its propensity to form nonlamellar structures, like the inverse hexagonal (H_{II}) phase (19,20). H_{II} phase-favoring phospholipids affect the structure and physical properties of membranes (8,21–23). These, in turn, can modulate the function of specific membrane proteins (24), an interaction illustrated by the requirement of a minimal proportion of hexagonal phase-preferring lipids for proper function of membrane-bound proteins, like rhodopsin in the visual system (25).

Other modifications to mitochondrial membrane composition during estivation also suggest greater bilayer stability. The mitochondrial membranes of estivating snails are characterized by an increased proportion of monoenes in PE. They also contain lower proportions of the H_{II} phase-preferring phospholipids, PE and CL (14). The other major constituent of mitochondrial membranes, PC, shows an increased average acyl chain length. Taken together, these changes suggest that membrane remodeling during estivation selects against H_{II} phase-preferring phospholipids, resulting in the adoption of a more stable lamellar phase in the phospholipid bilayer of estivators. H_{II} phase-preferring phospholipids are known to be important in a number of biological functions which are key to anabolic and catabolic processes, including trafficking of membrane fragments and proteins, fusion, and mitochondrial contact sites (8,24,26–29). The compositional changes which lower the tendency for formation of nonbilayer structures in mitochondrial membranes appear to be related to the depressed metabolism, and therefore reduced rates of these processes in estivating *Cepaea*.

Other aspects of the phospholipid compositions of mitochondria from estivatings snails are typical of those observed in association with reduced metabolic rates. The proportional content of monoenes in CL, PE, and PI was greater in estivating snails, due to the replacement of polyenes, like 18:2n-6 and 18:3n-3, with 16:1 and 18:1. Similarly, increased proportions of phospholipid monoenes have been shown to correlate strongly with reduced metabolic rates in other organisms (30,31). The specific mechanism underlying this correlation remains unknown, and the effects of phospholipid incorporation of monoenes are complicated. While modeling studies indicate that greater incorporation of monoenes in the *sn*-1 position can induce looser packing arrangements (32), di-monoenoic phospholipids are better able to form highly ordered phases than di-polyenoic phospholipids (22). Thus, the neighboring fatty acyl chain will determine how increased monounsaturation affects phospholipid properties. The impact of increased monoene incorporation in PE, PI, and CL on membrane bilayer stability is, therefore, uncertain without positional information. The reported association between monoenes and metabolic rate in various systems is, however, an interesting phenomenon that warrants further investigation.

The n-3/n-6 ratio of membrane phospholipid fatty acids was reduced in estivating snails, primarily due to decreased proportions of 18:3n-3 and 18:4n-3 in PE and PC. Similarly, lower proportional contents of n-3 polyenes are characteristic of the mitochondrial membranes of animals with lower metabolic rates (30,31). This has been demonstrated allometrically in mammals (31) and through comparisons of reptiles with mammals (30). In contrast, higher levels of n-3 polyunsaturated fatty acids are associated with superior recovery from postischemia reperfusion in rat heart (33), when oxygen levels may be expected to be abnormally high. Thus, the significance of phospholipid n-3 content may be related to the cellular oxygen concentrations which characterize different metabolic rates and physiological states. Lower relative levels of n-3 polyenes in estivating snails thus appear to be related to the metabolic rate reduction in these animals.

Changes in phospholipid fatty acid composition induced by estivation may not be attributable solely to the effects of elongation or desaturation. Mitochondria of estivating snails contain fewer phospholipids than those of active animals. These phospholipids do not appear to be lost from the cell (14). This suggests that phospholipids may be removed from the mitochondria and sequestered elsewhere in the cell (14). We have observed that a significant reduction in total mitochondrial phospholipid content occurs during estivation. Preferential removal of specific phospholipid or fatty acid species from the membrane thus provides a mechanism for altering phospholipid-specific fatty acid composition which is independent of elongating and desaturating processes.

In summary, the dramatic changes in phospholipid-specific fatty acid composition demonstrated here are consistent with profound reductions in the rates of membrane-associated processes during estivation. These results also suggest that estivating snails are a valuable model for studies of the relationship between metabolic rate and mitochondrial membrane composition. The results of this study should be extended to vertebrate estivators and to investigations of the functional properties of mitochondrial and cellular membranes during estivation.

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