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Constancy of Liver Lipid Composition in Two Genera of Toads After a Short-Term Temperature Acclimation

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The lipid and fatty acid composition of *Bufo calamytes* and *Alytes obstetricans* livers has been studied. Data for both species are similar, and resemble closely those published for *Rana sculenta*. Total lipids constitute 20-25 % of the total liver dry weight; about one fifth of these are phospholipids; cholesterol makes up 1.7-2.2 % of the total liver dry weight. The most abundant phospholipids are phosphatidylcholine and phosphatidylethanolamine. The various lipid classes differ in their fatty acid composition: neutral lipids contain high proportions of palmitoleic and linoleic acids; phosphatidylcholine is characterized by its contents in palmitic acid, whereas cardiolipin contains polyunsaturated fatty acids. Thermal acclimation of toads for 96 h produces but few changes in liver lipid composition.

Only BARANSKA and WLODAWER (1) have explored the lipid composition of frog tissues *(Rana sculenta)*. The possibility of temperature acclimation of poikilotherms made us consider the anphibians as potential models for the study of the effect of temperature on membrane fluidity in vertebrates. An important body of evidence suggests an evolutionary adaptation of membranes to temperature (3), but experimentation in this field requires systems responding to relatively short-term acclimation periods, and this has been done only on micro-organisms or fish (see 7, and references therein).

Preliminary studies in this laboratory (F. M. GOÑI, unpublished) indicated the possibility of thermal acclimation of two genera of toads, namely *Bufo calamytes* and *Alytes obstetricans*. This acclimation was accompanied by changes in lipid composition of lung tissue and lung surfactant, which prompted us to study the lipid composition of livers from the temperature-acclimated toads. The corresponding results are summarized in this paper.

Materials and Methods

Bufo calamytes and *Alytes obstetricans* were caught at the end of the summer in Arija (Burgos, Northern Spain) and kept at 4°C without feeding. Hybernation was instaured under these conditions. Four groups of animals, about six of each species per group, were transferred to independent chambers at 4, 11, 20 and 27° C, with humidity higher than 90 %, and left for 96 h. The toads were anaesthetized with ether and killed by decapitation. The livers were immediately excised and homogenized in 0.25 M sucrose. Lipids were extracted according to SANTIAGO *et al.* (13). Total lipids were estimated microgravimetrically according to HUNTER and ROSE (8). Lipid phosphorus was determined in a fraction of the lipid extract according to BARTLETT (2). Phosphorus contents were multiplied by 25 in order to obtain an estimate of the amount of phospholipids.

The individual phospholipid classes were separated by thin-layer chromatography according to NESKOVIC and KOSTIC (9), or DITTMER and LESTER (4) and identified by comparison with standards, and through specific reactions (5). The spots were localized by exposing the plates to iodine vapours. Phosphorus contents of each spot were determined according to BARTLETT (2), and the amount of phospholipid calculated thereof.

Fatty acids were analyzed as their methyl esters by gas-liquid chromatography after transesterification in boron trifluoride-methanol (20 %, w/v) (5). Fatty acid methyl esters were separated in a Carlo Erba Fractovap 2350 gas chromatograph equipped with a flame ionisation detector. The column was filled with 10 % diethylenglycolsuccinate (DEGS) on Gas Chrom W, mesh 80/100. Carrier gas was N_2 at 20 ml/min. Oven temperature was 185° C, isothermal. The degree of unsaturation (D.U.) of a given sample was calculated from the data of percent fatty acid distribution as follows: D.U. = (% monoenes) + 2 (% dienes) + 3 (% trienes). The degree of unsaturation expressed in this way corresponds to the number of double bonds for 100 fatty acid molecules.

Chromatographic solvents were from Merck (Darmstadt) and were used without further purification. Gas-chromatographic supplies were from Xpectrix (Barcelona). Other chemicals were supplied by Sigma (Poole, Dorset).

Statistical significance was checked by means of the Student's «*t*-test».

Results

Total lipid, phospholipid and cholesterol contents of livers from toads acclim-

Table I. *Total lipids, phospholipids and cholesterol in livers of* Bufo calamytes *and* Alytes obstetricans *kept at various temperatures for 96 h.*
Data are expressed as mg/100 mg tissue dry weight ± S.E.M. of three experiments.

Temperature (°C):	4	11	20	27
B. calamytes				
Total lipids	21.2 ± 4.6	25.1 ± 7.6	22.3 ± 4.4	20.4 ± 6.2
Phospholipids	5.4 ± 1.8	6.5 ± 1.3	5.8 ± 1.5	5.5 ± 1.7
Cholesterol	1.9 ± 0.1	2.2 ± 0.2	1.7 ± 0.2	1.8 ± 0.2
A. Obstetricans				
Total lipids	25.5 ± 2.6	24.0 ± 0.8	24.7 ± 1.4	22.3 ± 0.8
Phospholipids	4.5 ± 0.7	4.0 ± 0.8	4.1 ± 0.8	5.2 ± 0.5
Cholesterol	1.7 ± 0.2	1.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.1

Table II. *Percent distribution of main phospholipid classes in the lipid extracts of* B. calamytes *and* A. obstetricans *livers.* The toads had been kept for 96 h at various temperatures. Figures correspond to mean values ± S.E.M. (n = 3).

	B. calamytes				A. obstetricans			
Temperature (°C):	4	11	20	27	4	11	20	27
Lysoderivatives	4.2±2.3	6.5±2.0	7.5±0.9	7.7±0.8	15.6±4.6	12.3±1.9	11.5±1.4	4.7±1.3
Phosphatidylserine	10.7±1.2	13.0±2.1	11.8±1.9	12.2±2.2	12.4±2.0	10.7±3.4	15.1±1.8	11.3±1.9
Sphyngomyelin	10.9±3.5	10.5±0.0	13.5±0.7	11.0±2.2	17.7±2.0	13.3±2.5	14.5±0.5	15.7±2.8
Phosphatidylinositol	4.0±1.0	6.8±0.5	6.8±1.7	7.7±2.4	6.3±0.9	7.3±0.5	4.2±1.0	4.6±0.9
Phosphatidylcholine	31.6±6.9	29.8±3.2	27.9±6.5	25.1±1.3	16.4±5.7	18.3±5.3	21.9±0.0	31.8±4.4
Phosphatidylethanolamine	18.4±2.8	14.4±3.0	12.2±1.1	18.5±3.9	13.9±4.5	14.4±3.2	16.0±0.3	17.3±3.0
Cardiolipin	11.5±5.5	9.1±0.2	12.3±2.1	8.7±2.6	11.2±1.6	11.8±2.6	10.0±1.3	7.5±3.3
Phosphatidyl-N,N-dimethyl-ethanolamine	11.9±1.5	7.6±0.0	14.3±4.7	8.7±2.6	8.7±3.9	8.7±3.6	7.4±2.5	7.5±3.3

Table III. *Percent distribution of the main fatty acid of total lipids and lipid fractions of* B. calamytes *and* A. obstetricans *livers.* The toads had been kept for 96h at various temperatures. Data correspond to mean values of three experiments.

Temperature (°C):	Bufo calamytes									Alytes obstetricans								
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:4	D.U.	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:4	D.U.
Total lipid																		
4	3.2	27.5	6.2	7.5	26.6	14.7	5.3	8.8	113	1.5	22.4	11.2	8.2	26.1	17.4	6.4	9.3	128
11	4.5	32.6	8.5	8.0	23.3	16.4	6.9	7.6	115	0.9	17.0	8.4	10.2	26.0	19.0	6.3	11.8	138
20	1.5	24.0	7.3	8.5	25.6	17.6	4.4	10.8	124	0.7	24.6	8.0	9.2	26.5	16.3	5.7	9.6	122
27	2.8	24.8	7.9	8.4	25.2	16.8	3.2	10.6	118	1.4	27.0	7.1	7.9	28.7	13.7	6.6	8.2	115
Non-polar lipid																		
4	7.3	27.8	11.5	3.5	29.6	14.5	5.2		85	3.6	27.5	16.3	4.8	27.4	13.0	5.2		91
11	5.3	26.7	12.5	3.1	36.1	11.3	4.5		84	3.5	28.0	11.1	3.7	30.2	14.0	9.3		97
20	4.4	21.0	13.4	3.1	38.8	15.9	3.1		93	3.6	29.3	9.2	5.8	31.3	14.5	6.5		89
27	6.5	23.0	13.1	2.6	34.8	17.0	2.6		89	2.5	26.0	12.8	4.6	36.0	11.5	6.2		90
Sphyngomyelin																		
4		46.9	3.4	25.3	15.0	9.1			36		35.2	5.0	18.9	24.7	1.6			32
11		51.5	2.3	24.4	15.0	6.7			30		34.1	6.5	26.0	18.2	14.9			54
20		46.0	3.2	25.4	16.0	8.7			36		39.0	8.1	32.4	10.8	9.5			37
27		46.0	3.9	23.3	21.8	4.5			34		37.8	5.1	27.5	21.5	7.8			42
Phosphatidylcholine																		
4		64.1	3.7	7.4	14.4	10.0			38		58.4	3.0	7.1	16.2	14.8			48
11		60.0	4.8	5.1	20.0	10.0			44		59.8	2.7	6.2	17.0	14.0			47
20		59.4	5.7	4.5	17.5	12.7			48		56.5	2.5	9.2	18.2	8.8			47
27		64.0	4.6	4.3	18.1	8.8			40		63.0	2.5	4.0	19.2	11.5			44
Phosphatidylethanolamine																		
4		37.2	5.5	16.3	28.7	11.6			57		33.4	5.1	16.2	28.0	17.5			68
11		36.3	3.6	18.1	27.4	14.1			59		36.0	3.0	17.9	26.9	16.2			62
20		44.0	5.3	29.6	14.0	6.8			32		25.2	3.7	10.7	48.3	11.8			75
27		37.8	6.6	20.0	29.2	6.5			48		24.3	4.7	18.2	32.2	20.3			77
Cardiolipin																		
4		37.6	1.5	16.4	24.6	15.1	4.6		70		35.8	5.7	14.5	20.0	18.0	5.7		78
11		37.3	1.7	18.6	24.8	14.0	4.8		68		38.8	2.6	13.8	23.1	16.4	5.9		76
20		32.5	1.7	18.3	23.4	19.4	4.9		78		37.5	3.3	18.9	19.5	15.8	4.9		69
27		29.0	1.1	16.7	23.6	24.5	4.9		88		46.9	1.5	21.6	15.5	10.9	3.3		48

ated at various temperatures for 96 h are shown in table I. In this context, «cholesterol» refers to any sterol reacting in the Liebermann-Burchard reaction, with cholesterol as standard. The amount of total lipid appears too high when compared with the phospholipid and sterol contents, even allowing a large proportion of triglycerides. Total lipid was estimated micro-gravimetrically and the lipid extract probably contains hydrophobic materials other than lipid, especially proteins soluble in chloroform-methanol. The results in table I do not reveal any significant differences between *Bufo* and *Alytes*, nor any variation that could be attributed to temperature acclimation.

The main phospholipid classes were separated by thin-layer chromatography and quantified as lipid phosphorus, as indicated in the Methods section. Mean results of three experiments are given in table II. The main phospholipids present were, in order of decreasing abundance, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphyngomyelin, cardiolipin, phosphatidyl-N, N-dimethylethanolamine and phosphatidylinositol. Statistical analysis failed to reveal any significant differences between species, or attributable to temperature acclimation.

As a further attempt to characterize the lipids present in *B. calamytes* and *A. obstetricans* livers, the fatty acids associated with each lipid fraction were analyzed by gas-liquid chromatography (table III). The main fatty acids found in the total lipid extract are myristic (C 14:0), palmitic (C 16:0), palmitoleic (C 16:1), stearic (C 18:0), oleic (C 18:1), linoleic (C 18:2), linolenic (C 18:3) and arachidonic (C 20:4). Of these, myristic is only found in appreciable amounts (>1 %) in neutral lipids, whereas linolenic appears only in neutral lipids and in the phospholipid cardiolipin. Arachidonic acid is not found in any of the lipid fractions studied by gas-liquid chromatography and presumably it is associated with phosphatidylserine or phosphatidylinositol. The various lipid classes differ in their fatty acid composition: neutral lipids are characterized by their relatively high proportion of palmitoleic and linoleic acids, and low levels of stearic acid; sphyngomyelin contains the highest proportion of stearic and the lowest of linoleic acid among the phospholipids; the main feature of phosphatidylcholine fatty acid composition is the high proportion of palmitic acid, up to 64 % in some cases; phosphatidylethanolamine shows a somewhat intermediate fatty acid composition among the different phospholipid classes; finally, cardiolipin is characterized by the high contents in polyunsaturated fatty acids. These differences in the percent distribution of fatty acids lead to variation in the degree of unsaturation of the individual lipid classes: phospholipid unsaturation increases in the order sphyngomyelin, phosphatidylcholine, phosphatidyletanolamine, cardiolipin; neutral lipids are also highly unsaturated.

The study of the fatty acids associated with the different lipid species reveals some differences between *Bufo* and *Alytes*, as well as come minor changes that could be attributed to tempertaure acclimatation. For instance, total lipid is higher in *Alytes*, but only after acclimatation at 4 or 11° C; the same effect, but at 20 or 27° C is found in phosphatidylethanolamine fatty acids; in cardiolipin, *Alytes* is more unsaturated than *Bufo* at 4 and 11° C, but the unsaturation of *Alytes* cardiolipin decreases considerably with temperature.

Discussion

The purpose of this investigation was, on one hand, to establish the lipid composition of the liver of the amphibians *Alytes obstetricans* and *Bufo calamytes*, and on the other, to explore the possibility of altering that lipid composition through short-term temperature acclima-

tion. Both aspects will now be discussed separately.

BARANSKA and WLODAWER (1) have studied the total fatty acids in the liver of frog *(Rana sculenta)*. Our results are practically the same as theirs (table III), except that toads contain about 8 % more oleic (C 18:1) acid, and no tetraicosenoic acid. Fatty acids from neutral lipids are also very similar in *Rana*, *Alytes* and *Bufo*, but toads, according to the present data, have a higher proportion of myristic (C 14:0) acid. BARANSKA and WLODAWER (1) did not report the fatty acid composition of the individual phospholipid classes of *Rana*, but the similarities found in total and neutral lipid fatty acids in *Rana*, *Alytes* and *Bufo* support our observations on the basic similarities in lipid composition of both genera of toads.

Our data agree in general with those published for mammal or fish liver (12, 15). The same can be said of our observations on the percent phospholipid distribution: phosphatidylcholine and phosphatidylethanolamine are the most abundant membrane phospholipids in all eukaryotic organisms (12). The high unsaturation of cardiolipin with respect to phosphatidylcholine is also found in mammals (10) but not in yeasts (6, 11).

When a poikilotherm is subjected to a relatively long-term stress, adaptative changes are initiated in the form of physiological compensations. This process is referred to as «thermal acclimation» (7). It has been shown in many instances that thermal acclimation has pronounced effects on the lipid composition of poikilotherms. Cold temperatures are generally associated with an increased degree of unsaturation, this phenomenon being explained as «homeosviscous adaptation» (14). The possibility of long-term temperature acclimation in amphibians has been shown by BARANSKA and WLODAWER (1), acclimated frogs at various temperatures for 6-8 weeks: acclimation at low temperatures was accompanied by an increase in total lipid unsaturation, mainly as a consequence of an increase in polyunsaturated fatty acids. As we have seen (table III), total fatty acids are not affected in toads after a short acclimation time (96 h), although in some cases, such as in *Alytes* cardiolipin, some adaptation may have taken place even after this brief period. Other results from this laboratory (F. M. GOÑI, to be published) show unequivocal thermal acclimation of toad lung lipids after 96 h. This suggests that thermal acclimation in amphibians takes place at different stages in the various organs, and that studies including acclimation periods of varying lenghts, between 3-4 days and 6-8 weeks, are required for a full understanding of the process.

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Resumen

Se estudia la composición lipídica y los ácidos grasos en hígado del sapo *Bubo calamytes* y *Alytes obstetricans*. Los datos de ambas especies son similares y recuerdan a los publicados de *Rana sculenta*. Los lípidos constituyen un 20-25 % del peso seco de hígado; aproximadamente una quinta parte son fosfolípidos; el colesterol constituye un 1,7-2,2 % del peso seco de hígado. Los fosfolípidos más abundantes son la fosfatidilcolina y la fosfatidiletanolamina. Las distintas clases de lípidos poseen diferente composición en ácidos grasos: los lípidos neutros contienen gran proporción de ácidos linoleico y palmitoleico; la fosfatidilcolina se caracteriza por su alto contenido en ácido palmítico, mientras que la cardiolipina contiene muchos ácidos grasos poliinsaturados. La aclimatación de los sapos a distintas temperaturas durante 96 h apenas produjo cambios en la composición lipídica de sus hígados.

References

1. BARANSKA, J. and WLODAWER, P.: *Comp. Biochem. Physiol.*, 28, 553-570, 1969.
2. BARTLETT, G. R.: *J. Biol. Chem.*, 234, 446-462, 1959.
3. COSSINS, A. R. and PROSSER, C. L.: *Proc. Natl. Acad. Sci. USA*, 71, 522-525, 1978.
4. DITTMER, J. C. and LESTER, R. L.: *J. Lipid Res.*, 5, 126, 1964.
5. GOÑI, F. M. and MACARULLA, J. M.: *Laboratorio*, 384, 501-519, 1978.
6. GOÑI, F. M., MUÑOZ, D., GÓMEZ, J. C. and SANTIAGO, E.: *An. Quim.*, 74, 516-518, 1978.
7. HAZEL, J. and PROSSER, C. L.: *Physiol. Rev.*, 54, 620-627, 1974.
8. HUNTER, K. and ROSE, A. H.: *Biochim. Biophys. Acta*, 260, 629-653, 1972.
9. NESKOVIC, N. M. and KOSTIC, D. M.: *J. Chromatography*, 35, 297-300, 1968.
10. PÉREZ, P., LÓPEZ-MORATALLA, N. and SANTIAGO, E.: *Rev. esp. Fisiol.*, 29, 239-246, 1973.
11. PONTÓN, J., REGÚLEZ, P., DOMÍNGUEZ, J. B., GOÑI, F. M. and URUBURU, F.: *Current Microbiol.*, 3, 327-333, 1980.
12. QUINN, P. J.: In «Molecular Biology of Cell Membranes», MacMillan, London, 1976.
13. SANTIAGO, E., MULE, S., REDMAN, M., HOKIN, M. R. and HOKIN, L. E.: *Biochim. Biophys. Acta*, 84, 550-562, 1964.
14. SINENSKY, M.: *Proc. Natl. Acad. Sci. USA*, 75, 522-525, 1974.
15. WODTKE, E.: *Biochim. Biophys. Acta*, 529, 280-291, 1978.