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# THE EFFECT OF ACCLIMATION TEMPERATURE ON THE ACTIVATION ENERGIES OF STATE III RESPIRATION AND ON THE UNSATURATION OF MEMBRANE LIPIDS OF GOLDFISH MITOCHONDRIA

GUIDO VAN DEN THILLART and JAN MODDERKOLK

*Zoological Laboratory, Department of Animal Physiology, State University of Leiden, Kaiserstraat 63, Leiden (The Netherlands)*

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## Summary

The influence of the acclimation temperature on the thermotropic behaviour of mitochondrial respiration and on the degree of unsaturation of mitochondrial membrane lipids has been studied. The mitochondria were isolated from red muscle, white muscle and liver of goldfish acclimated to 5, 20 and 30°C. ADP-activated succinate oxidation was measured at different temperatures and resulted in non-linear Arrhenius-plots with breaks between 10 and 23°C. As for the break-temperatures, there was found a shift downwards in preparations of decreased acclimation temperatures. This could be caused by a changed composition of membrane lipids and a simultaneous shift of the membrane phase transition temperature. Therefore, the fatty acid composition of all membrane preparations was analyzed. However, no consistent change of the degree of unsaturation due to a changed acclimation temperature could be found.

## Introduction

In biomembrane research several studies with microorganisms demonstrated a correlation between thermotropic phase changes and breaks in Arrhenius plots of membrane-bound enzymes. The break temperature at which these phase changes occur was found to be influenced by the relative concentrations of fatty acids in the growth medium, thus providing a mechanism for controlling the membrane fluidity [1]. It has been demonstrated that transition temperatures and breaks in Arrhenius plots can be shifted simultaneously by changing the degree of unsaturation of membrane lipids [2—4].

Because proper functioning of biomembranes below the transition tempera-

Abbreviation: EGTA, ethyleneglycol bis(α-aminoethylether)-*N*,*N*'-tetraacetic acid.

ture will become difficult, it is evident that poikilothermic organisms should adapt their temperature-sensitive membranes to changed environmental temperatures. It has been demonstrated that acclimation of poikilothermic vertebrates to lower temperatures is related to an increase of unsaturated membrane lipids [5—8], with the exception of fish muscle lipids [9]. There is also some evidence with respect to membrane-bound enzymes, that the acclimation temperature influences biomembranes, Smith [10] and Lyons and Raison [11] working with cold acclimated fish could not find breaks in Arrhenius plots of mitochondrial respiration, while Irving and Watson [12] indeed observed breaks in Arrhenius plots of succinate oxidase activity from tropical fish. With muscle mitochondria of carp, Wodtke [13] observed a shift of the break-temperature in Arrhenius plots of succinate oxidase due to a change of acclimation temperature. However, Irving and Watson [12] not only found breaks in Arrhenius plots of succinate oxidase at room temperature, but they also observed that the mitochondrial membrane lipids were highly unsaturated. This is hard to understand, for at room temperature no phase transition will occur in such unsaturated membranes. From membrane studies, using spin labels and differential scanning calorimetry, it was found that breaks in Arrhenius plots and membrane phase transitions were not always correlated [14—16]. Therefore, it appears that the shift of the break temperature in Arrhenius plots is not necessarily caused by a change in the unsaturation of the membrane lipids.

In this study, with goldfish mitochondria from different tissues, we found some evidence that breaks in Arrhenius plots of state IH respiration are not correlated with the degree of unsaturation of membrane lipids.

## Terminology

The thermotropic phase transition, changing the liquid crystalline structure of membrane lipids into a gel-like mesophase, will be referred to as membrane phase transition. Breaks in Arrhenius plots, indicating a change in activation energy caused by a transition of the protein structure, will only be referred to as breaks in order to prevent confusion.

## Materials and Methods

*Conditions.* Healthy goldfish (±100 g) were kept for at least 2 months at 20° C before acclimation. Two groups of 10 fish and one group of 20 fish were acclimated to 5, 20 and 30° C, respectively. The acclimation levels were reached by a temperature increase of 1.0° C per day. The fish were fed with a mixture of liver, spleen, wheat germs, yeast and cod-liver oil (10 : 2 : 1 : 0.5 : 0.8, w/w) and kept at the acclimation level (±0.1° C) for a minimum of 5 weeks in tanks with running tap water and oxygen levels between 80 and 90% air saturation.

*Preparations of mitochondria.* The animals were killed by decapitation and the tissues were rapidly excised. The liver was gently homogenized in a Potter-Elvehjem type homogenizer in a solution of 210 mM mannitol/70 mM sucrose/ 10 mM ethyleneglycol bis($\alpha$-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA)/ 0.2% bovine serum albumin/100 mM Tris · HCl, pH 7.4, (at 3° C). The Teflon pestle of the homogenizer had a diameter 0.25—0.30 mm less than the internal

diameter of the tube [17]. Because the liver mitochondria sedimented at 1000 $\times g$, the suspension was centrifuged at 250 $\times g$ for 15 min and the supernatant recentrifuged at 10 000 $\times g$ for 10 min in a Sorvall-RC-2B superspeed refrigerated centrifuge. The resultant pellet was resuspended in 0.5—1.0 ml of the same medium and stored in ice until use. The red muscle (musculus lateralis superficialis) and the white muscle (musculus dorsalis profundus) were minced into 1 mm cubes with a precooled stainless steel tissue slicer. We used a modification of the mitochondria isolation procedure of Bullock, Carter and White [18]. The procedure described below, was specially adapted to fish muscle, and yielded higher respiratory control ratios. With succinate we found respiratory control ratios: 5 ± 1 and with glutamate: 15 ± 5. In testing the isolation procedure we found that the protease trypsin provided better results than nagarse and subtilisin. A 25% suspension of minced muscle in a solution of 210 mM mannitol/70 mM sucrose/10 mM EGTA and 100 mM Tris · HCl, pH 7.4 (at 3° C), was partly digested by trypsin (0.5 mg/g tissue). After 10 min incubation at 1° C, without stirring to prevent damage, the suspension was made 5% (w/w) and bovine serum albumin was added to a final concentration of 0.2%. The suspension was then homogenized as described, centrifuged at 1000 $\times g$ for 15 min to remove cell debris and recentrifuged at 10 000 $\times g$ for 10 min. The pellet was resuspended in 0.5—1.0 ml of the same medium with 0.2% albumin.

*Mitochondrial respiration.* Oxygen consumption by mitochondria was measured with a Clark-type electrode and a biological oxygen monitor (Yellow Springs Instruments). The reaction vessel was thermostated with a 10 l Tamson waterbath and a Colora dip-cooler. Two instruments were employed simultaneously, one operating from 5 to 15° C and the other from 20 to 40° C. Full scale adjustments were made with air-equilibrated distilled water at the experimental temperature. The oxygen content at each temperature was exactly determined by the Winkler titration method. The reaction medium consisted of 200 mM mannitol/70 mM sucrose/10 mM EGTA/10 mM $KH_2PO_4$/10 mM sodium-succinate/100 mM Tris · HCl, pH 7.4 (at experimental temperature). The oxygen consumption of 100 µl mitochondrial suspension in 1.40 ml medium was measured before and after addition of 140 nmol ADP to the reaction vessel. ADP was added in 25-µl aliquots by means of a calibrated microburette. Respiratory control ratios and ADP oxygen consumption ratios were calculated as described by Chance and Williams [19] and Estabrook [20]. ADP purity was determined enzymatically according to van den Thillart et al. [21].

*Cytochrome c oxidase.* The enzyme activity was measured at 23° C according to the spectroscopic method of Caldwell [35] and Smith [23]. It was found that auto-oxidation of reduced cytochrome *c* could be depressed from 1.8 to 0.4% per h, by bubbling with $N_2$ during 30 min. This procedure resulted in an almost 100% reduction of cytochrome *c*. After addition of 0.5 mg Triton X-100 per mg protein [24], homogenates and mitochondria suspensions were diluted in a single step to a proper concentration, This was done because some activity was lost in each dilution step. Cytochrome *c* oxidase activity measurements were performed to calculate the yield of the mitochondria isolation procedure, since cytochrome *c* oxidase is a marker of the inner mitochondrial membrane. This enabled us to express mitochondrial respiration as nmol $O_2 \cdot min^{-1} \cdot g^{-1}$.

*Electron microscopy.* After isolation as described above, the sedimented mitochondria were fixed by a solution of 100 mM cacodylate, pH 7.4/0.8% glutaraldehyde/0.67% $OsO_4$/165 mM mannitol/55 mM sucrose. Mannitol and sucrose were added to obtain the same osmolarity, thus preventing osmotic shock. After 30 min a second fixation was performed with a solution of 1% $OsO_4$ in 100 mM cacodylate buffer, pH 7.4, for 75 min. After dehydration the pellets were embedded in epon. Thin films (±100 Å) were contrasted with uranyl-acetate and lead-citrate and examined with a Philips E.M. 300.

*Fatty acid analysis.* Mitochondria suspensions were freeze-dried and stored at −35° C. The lipids were extracted from the stored material according to Folch et al. [25]. After saponification, the fatty acids were methylated according to Kluytmans [26]. Methyl esters were analysed with a Becker 2300 gas chromatograph with flame ionisation detection. Separation was performed at 180° C on chromosorb-W coated with 20% polyethylene glycol adipate + 2% $H_3PO_4$. Identification was achieved by external standards, grafical interpolation methods and by hydrogenation of the unsaturated lipids [27].

## Results

*Mitochondrial respiration.* Mitochondria were isolated from red muscle, white muscle and liver of goldfish acclimated to 5, 20 and 30° C. The isolation procedure was reproducable and yielded mitochondria with high respiratory control ratios (Table I), indicating a strongly depressed state IV respiration. The phosphorylation capacity of the mitochondria, expressed by the molar ratio between added ADP and consumed oxygen reached in all muscle preparation the theoretical value 2.0 for succinate oxidation. In liver mitochondria the ADP/O ratio reached lower values. The respiratory control ratios of the liver preparations were also lower than those of the muscle preparations. These lower values observed in liver mitochondria could be caused by a high concentration of free fatty acids which are potent uncouplers of the oxidative phosphorylation. In all preparations the respiratory control ratios were decreased at the higher acclimation temperatures. There was however, no significant influence of the experimental temperature on the respiratory control ratio. There-

TABLE I

FUNCTIONAL CHARACTERISTICS OF ISOLATED MITOCHONDRIA

Mitochondria were isolated from red muscle, white muscle and liver of goldfish acclimated to 30, 20 and 5° C. State III (ADP-activated) and state IV (ATP-inhibited) respiration were measured with succinate as substrate. From these results the functional parameters ADP/O ratio and respiratory control ratio were calculated. The respiratory control ratio is defined by the activity in state III devided by the activity in state IV, and ADP/O by the amount of phosphorylated ADP-molecules per consumed oxygen atom.

| Acclimation temperature (° C) | ADP/O ratio * | | | Respiratory control ratio * | | |
|---|---|---|---|---|---|---|
| | Red muscle | White muscle | Liver | Red muscle | White muscle | Liver |
| 5 | 2.0 ± 0.2 | 2.0 ± 0.2 | 1.9 ± 0.2 | 6.0 ± 0.9 | 4.6 ± 1.1 | 3.1 ± 1.6 |
| 20 | 2.0 ± 0.2 | 2.0 ± 0.3 | 1.9 ± 0.2 | 4.7 ± 1.1 | 5.8 ± 1.0 | 4.0 ± 1.5 |
| 30 | 2.0 ± 0.2 | 2.0 ± 0.2 | 1.7 ± 0.2 | 4.9 ± 1.2 | 4.5 ± 0.5 | 2.9 ± 0.8 |

* Each condition comprises about 60 observations.

fore, it appears that mitochondria of warm-acclimated animals were more sensitive to the isolation procedure.

The isolation procedure was checked by electron microscopic examination. Relevant pictures of mitochondria isolated from red muscle, white muscle and liver are shown in Fig. 1. Morphological differences between the three types of

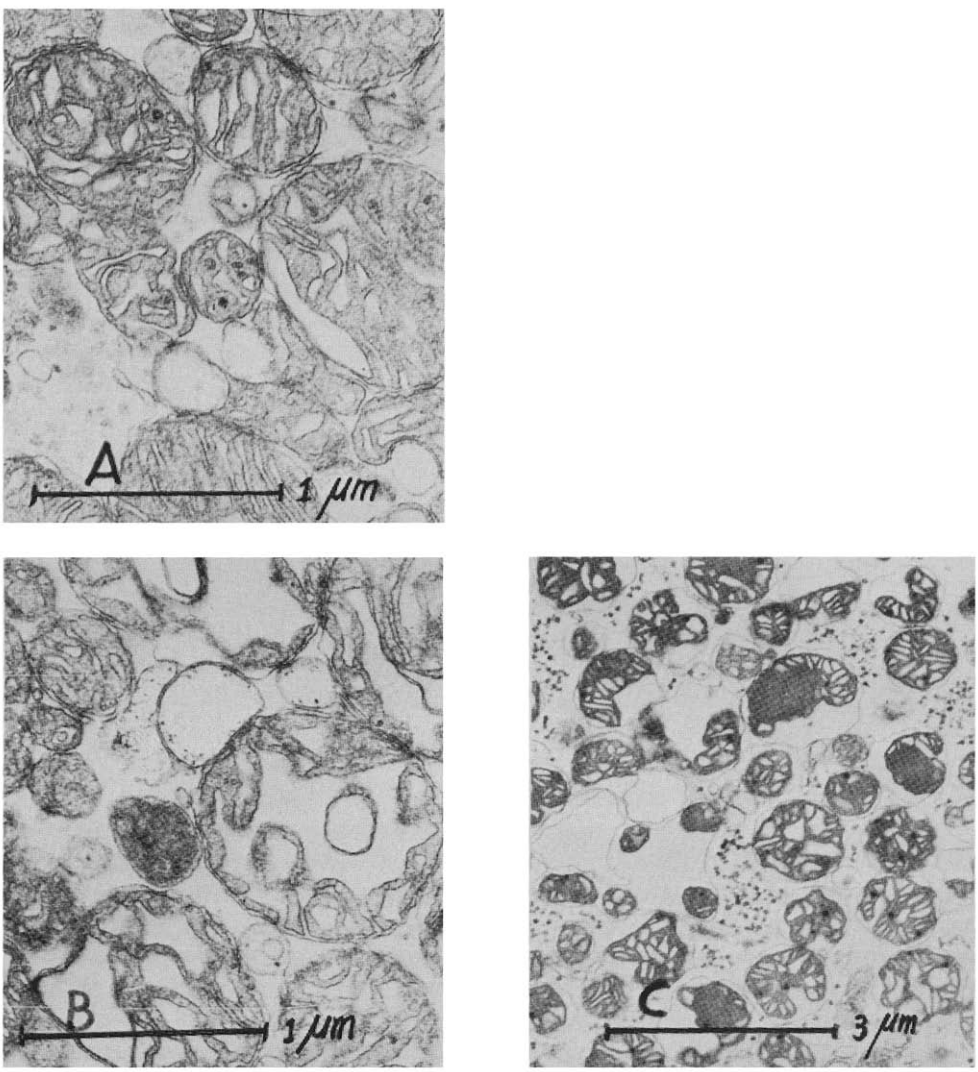


**Fig. 1. Electron microscopic photographs of mitochondria isolated from red muscle, white muscle and liver of goldfish. Mitochondria preparations were fixed by a medium containing 0.8% glutaraldehyde and 0.67% $OsO_4$ of the same osmolarity as the isolation medium to prevent an osmotic shock. A. Red muscle mitochondria are marked by densely packed inner membranes, indicating a high oxidative capacity. B. White muscle mitochondria have a smaller surface area and a large intercrystal space. C. Liver mitochondria have also a smaller surface area but are marked by a more dense and larger matrix. All pictures show condensed matrices and closed outer membranes, indicating the morphological intactness of the isolated mitochondria.**

mitochondria are obvious. Red muscle mitochondria (Fig. 1A) are marked by a strong enfolded inner membrane which indicates a high oxidative capacity; white muscle mitochondria (Fig. 1B) have a smaller inner membrane surface and a large intercristal space; liver mitochondria (Fig. 1C) have also a small inner membrane surface and are marked by a dense matrix. In all pictures the mitochondrial matrix is strongly condensed, obviously due to the hyperosmotic composition of the medium (500 mosM). The sharp boundaries and the occurrence of closed outer membranes indicate the intactness of the mitochondria; this is also underlined by the condensed configuration of the matrix, because any leak would have resulted in swollen matrices.

The yield of the isolation procedure was calculated from the specific activities of cytochrome *c* oxidase of tissue homogenates and of mitochondria suspensions. With that, the oxygen consumption of mitochondria were recalculated and expressed as units/g tissue. In Table II the natural logarithms of the oxygen consumption of mitochondria are given at 3 temperatures. A significant increase of the oxidative capacity was observed in muscle tissues due to a decrease of the acclimation temperature. The same phenomenon can be seen from the activities of cytochrome oxidase (Table III).

The natural logarithm of mitochondrial respiration measured at 10 temperatures was plotted versus $1/T$. Arrhenius plots from about 8 different preparations were normalised to give the same activities at the adaptation temperature. Statistical analysis of these plots revealed regression lines with correlation coefficients higher than 0.9. All Arrhenius plots obtained in this way are summarized in Fig. 2. In order to compare the influence of the acclimation temperature, we plotted the curves on a floating scale. The absolute activities expressed as ln units/g tissue are given in Table II. The break-temperatures (in °C) are indicated by arrows.

In the red-muscle mitochondria (Fig. 2) the influence of the acclimation temperature on the Arrhenius plots of mitochondrial respiration has two effects: first the break temperatures are shifted towards lower temperatures at the lower

TABLE II

TEMPERATURE DEPENDENCY OF MITOCHONDRIAL RESPIRATION

Mitochondria were isolated from red muscle, white muscle and liver of goldfish acclimated to 30, 20 and 5°C. State III (ADP-activated) respiration was measured at several temperatures. In this table the specific activities are given at 5.6, 20.3 and 30.0°C.

| Acclimation temperature (°C) | Tissue | *n* | Oxygen consumption ln(nmoles $O_2 \cdot min^{-1} \cdot g^{-1}$ tissue) | | |
|---|---|---|---|---|---|
| | | | 5.6°C | 20.3°C | 30.0°C |
| 30 | Red muscle | 6 | 4.77 ± 0.81 | 6.42 ± 0.81 | 6.87 ± 0.81 |
| | White muscle | 7 | 3.90 ± 0.33 | 5.27 ± 0.33 | 5.73 ± 0.32 |
| | Liver | 6 | 2.50 ± 0.53 | 4.63 ± 0.52 | 5.16 ± 0.49 |
| 20 | Red muscle | 6 | 4.81 ± 0.73 | 6.38 ± 0.71 | 6.68 ± 0.71 |
| | White muscle | 6 | 4.26 ± 0.31 | 5.57 ± 0.27 | 5.97 ± 0.28 |
| | Liver | 5 | 2.46 ± 0.46 | 4.49 ± 0.42 | 4.85 ± 0.43 |
| 5 | Red muscle | 8 | 5.52 ± 0.55 | 6.72 ± 0.54 | 7.28 ± 0.59 |
| | White muscle | 7 | 4.33 ± 0.54 | 5.56 ± 0.55 | 6.01 ± 0.55 |
| | Liver | 8 | 3.62 ± 0.38 | 4.85 ± 0.42 | 5.23 ± 0.42 |

TABLE III

CYTOCHROME *c* OXIDASE ACTIVITY, MEASURED AT 23°C, OF TISSUES PREPARED FROM GOLDFISH ACCLIMATED AT 30, 20 AND 5°C

| Tissue | Acclimation temperature (°C) | *n* | Cytochrome *c* oxidase ($10^2$ μmol · $min^{-1}$ · $g^{-1}$) | Groups in *t*-test * | *P* (%) |
|---|---|---|---|---|---|
| Red muscle | 5 | 13 | 7.0 ± 2.6 | 5°—20°C | <1.0 |
| | 20 | 12 | 3.9 ± 1.4 | 5°—30°C | <0.5 |
| | 30 | 10 | 2.6 ± 1.4 | 20°—30°C | <5 |
| White muscle | 5 | 13 | 1.4 ± 0.7 | 5°—20°C | <5 |
| | 20 | 12 | 1.0 ± 0.5 | 5°—30°C | <2.5 |
| | 30 | 10 | 0.6 ± 0.4 | 20°—30°C | >5 |
| Liver | 5 | 12 | 1.0 ± 0.5 | 5°—20°C | >5 |
| | 20 | 5 | 1.1 ± 0.3 | 5°—30°C | >5 |
| | 30 | 9 | 0.8 ± 0.2 | 20°—30°C | >5 |

* Single tail probability is tested according to Student's *t*-test.

acclimation temperatures and second at the acclimation temperature of 5° C the Arrhenius plots tend toward more linear curves. Also in the curves of the white muscle mitochondria we observed the same two effects; the shift of the break temperature is even larger.

In contrast to the muscle preparations the liver mitochondria of the three acclimation temperatures show little differences. There is no shift of the break temperature and activation energies below and above the break-temperature were similar at all acclimation temperatures (Table IV). However, at the acclimation temperature of 5° C a second break in the curve was observed at 11.9° C, below this temperature a low activitation energy was found, i.e., 6.9 kcal/mol.

Thus, we have found that in all mitochondria preparations the Arrhenius plots of state III respiration were non linear: activation energies above the break-temperature were about 7 kcal/mol and below, about 17 kcal/mol (Table IV). A downward shift of the break-temperature due to a lowered acclimation temperature was observed in red- and white-muscle mitochondria but, however, not in liver mitochondria. A significant change of the activities of mitochondrial respiration (Table II) as well as cytochrome *c* oxidase (Table III) was observed in muscle mitochondria, not in liver mitochondria.

*Fatty acid composition of mitochondrial membrane lipids.* The fatty acid composition of mitochondrial membrane lipids of goldfish acclimated to 5, 20 and 30° C is given in Table V. In these preparations there is clearly not a shift towards unsaturation, due to decreased environmental temperature. Generally the unsaturation index is used for the sake of comparison. This index is the summation of all double bounds (Table VI). We may compare the index of mono- and polyunsaturated fatty acids. As can be seen, the amount of monounsaturated bounds is rather stable, therefore, as a simplification we compare polyunsaturated indices.

In all tissues the lowest value is found at acclimation temperature of 20° C. In both white- and red-muscle preparations we found the highest value at 5° C; however, liver preparation had the highest index at 30° C. It must be mentioned

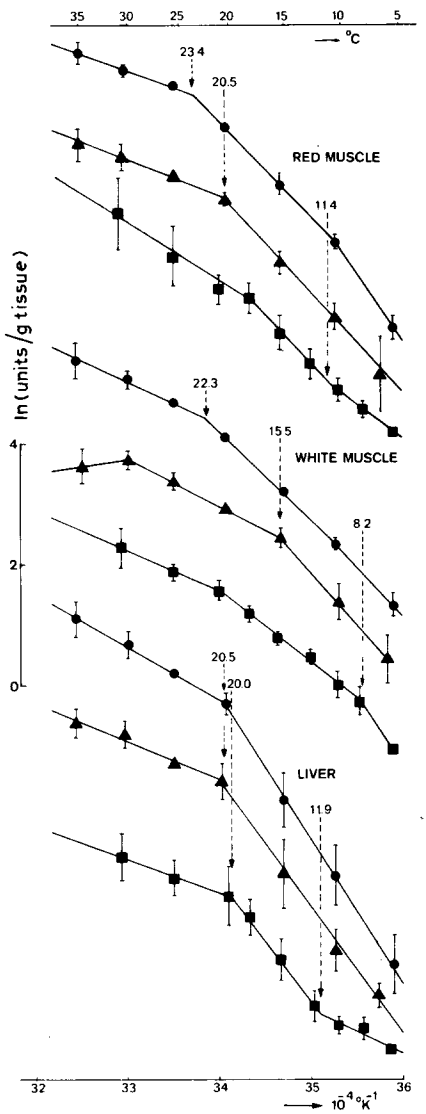


**Fig. 2. Arrhenius plots of mitochondrial state III respiration. The natural logarithm of the respiration is plotted on a floating scale versus $1/T$. The absolute activities are given in Table II. Mitochondria were isolated from red muscle, white muscle and liver of goldfish acclimated to 30° C (●———●), 20° C (▲———▲) and 5° C (■———■). Each curve is composed from the results of approx. 8 different preparations; the lines were fitted by regression analysis. The standard deviation in each point is indicated by a vertical bar and the break temperature (° C) in each curve by an arrow.**

that there are remarkable tissue-specific differences; the lowest values were found in red muscle and the highest values in white muscle.

Because food lipids are sometimes reflected in the composition of body lipids we determined its fatty acid composition too (Table V). Although 33 different fatty acids were found with peak area 0.1% or greater, 71% consists of only 6 fatty acids (16 : 0, 16 : 1, 18 : 1, 18 : 2, 20 : 5, 22 : 6). The composi-

TABLE IV

BREAK TEMPERATURES AND ACTIVATION ENERGIES FOR STATE III RESPIRATION OF MITOCHONDRIA ISOLATED FROM GOLDFISH ACCLIMATED TO 5, 20 AND 30°C

| Acclimation temperature (°C) | Tissue | $n$ | Break-temperature (°C) | | Activation energy (kcal/mol) * | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $T_1$ | $T_2$ | Below ($T_1$) | Between ($T_1$ and $T_2$) | Above ($T_1$) | Above ($T_2$) |
| 30 | Red muscle | 6 | 23.4 | — | 17.0 | — | 5.1 | — |
| | White muscle | 7 | 22.3 | — | 14.6 | — | 7.3 | — |
| | Liver | 6 | 20.5 | — | 23.5 | — | 8.9 | — |
| 20 | Red muscle | 6 | 20.5 | — | 16.9 | — | 5.8 | — |
| | White muscle | 6 | 15.5 | — | 17.2 | — | 7.6 | — |
| | Liver | 5 | 20.5 | — | 20.9 | — | 5.5 | — |
| 5 | Red muscle | 8 | 17.7 | 11.4 | 12.1 | 15.4 | — | 10.3 |
| | White muscle | 7 | 20.5 | 8.2 | 18.1 | 11.2 | — | 8.5 |
| | Liver | 8 | 20.0 | 11.9 | 6.8 | 18.5 | — | 6.9 |

* The standard deviation of all activation energies is ±2.0 kcal/mol.

TABLE V

FATTY ACID COMPOSITION OF MITOCHONDRIAL MEMBRANE LIPIDS

Mitochondria were isolated from red muscle, white muscle and liver of goldfish acclimated to 30, 20 and 5°C. Suspensions were used for both respiration measurements and lipid extraction. The fatty acids were analyzed after hydrolysis of the membrane lipids; the composition is expressed as mol%.

| Fatty acid shorthand name | Red muscle | | | White muscle | | | Liver | | | Diet lipids |
|---|---|---|---|---|---|---|---|---|---|---|
| | 5°C | 20°C | 30°C | 5°C | 20°C | 30°C | 5°C | 20°C | 30°C | |
| 14:0 | 1.2 | 1.4 | 1.4 | 0.7 | 1.0 | 1.0 | 1.3 | 1.7 | 0.8 | 4.8 |
| 14:1 | — | — | — | — | — | — | — | — | — | 0.5 |
| 14:2 | — | — | — | — | — | — | — | — | — | 0.4 |
| 15:0 i | 0.3 | 0.4 | 0.4 | 0.3 | 0.2 | 0.2 | 0.3 | 0.4 | 0.1 | 0.2 |
| 15:0 ai | 0.3 | 0.2 | 0.4 | 0.3 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 |
| 15:0 | 0.5 | 0.5 | 0.5 | 0.4 | 0.5 | 0.4 | 0.6 | 0.7 | 0.4 | 0.1 |
| 15:2 | — | — | — | — | — | — | — | — | — | 0.6 |
| 16:0 i | 0.2 | 0.3 | 0.2 | 0.2 | 0.5 | 0.2 | 0.4 | 0.5 | 0.2 | 0.2 |
| 16:0 | 10.6 | 14.4 | 10.7 | 13.8 | 16.5 | 13.6 | 17.3 | 19.1 | 15.3 | 11.3 |
| 16:1 | 4.2 | 4.3 | 4.5 | 2.7 | 2.5 | 3.2 | 2.6 | 2.7 | 1.8 | 9.6 |
| 16:2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 | — | 0.2 |
| 17:0 i | 0.8 | 0.6 | 0.5 | 0.7 | 0.6 | 0.6 | 1.6 | 1.5 | 1.5 | — |
| 17:0 ai | 0.6 | — | 0.3 | — | — | 0.3 | 1.1 | 1.2 | 0.8 | 0.2 |
| 17:0 | 0.6 | 0.5 | 0.5 | 0.7 | 0.6 | 0.6 | 0.9 | 1.0 | 1.3 | 0.5 |
| 17:1 | 0.5 | 0.5 | 0.5 | 0.3 | 0.4 | 0.4 | 0.3 | 0.3 | 0.2 | 0.6 |
| 18:0 i | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | — |
| 18:0 ai | — | — | — | — | — | — | — | — | — | 0.5 |
| 18:0 | 5.0 | 7.7 | 5.0 | 7.1 | 9.4 | 7.2 | 8.2 | 7.0 | 11.0 | 2.1 |
| 18:1 | 20.5 | 19.8 | 23.4 | 13.1 | 14.8 | 17.3 | 15.4 | 17.4 | 18.0 | 15.5 |
| 18:2 | 14.5 | 16.6 | 14.8 | 9.3 | 13.1 | 10.7 | 6.5 | 9.9 | 6.0 | 18.2 |
| 18:3 | 3.5 | 3.3 | 2.9 | 1.7 | 2.2 | 2.0 | 1.4 | 1.6 | 1.0 | 4.9 |
| 19:0 | 1.0 | 0.5 | 0.5 | 0.5 | 0.5 | 0.1 | 0.8 | 0.1 | 0.4 | — |
| 19:1 | 0.8 | 0.9 | 0.8 | 0.5 | 0.5 | 0.6 | — | 0.4 | 0.4 | 0.6 |
| 20:0 i | — | — | — | — | — | — | — | — | — | 0.2 |
| 20:0 | — | — | — | — | — | — | — | 0.4 | 0.4 | — |
| 20:1 | 4.1 | 3.1 | 4.0 | 2.8 | 2.6 | 3.2 | 7.6 | 3.1 | 1.5 | 0.9 |
| 20:2 | 0.7 | 0.7 | 0.5 | 0.6 | 0.8 | 0.4 | 0.9 | 1.0 | 0.6 | — |
| 20:3 | 2.4 | 2.3 | 2.1 | 2.2 | 2.3 | 1.9 | 1.5 | 1.8 | 1.8 | 1.1 |
| 20:4 | 6.0 | 6.3 | 5.8 | 9.1 | 7.3 | 7.7 | 7.0 | 5.9 | 7.4 | 2.4 |
| 20:5 | 3.8 | 2.5 | 3.7 | 4.5 | 2.5 | 4.1 | 3.1 | 2.8 | 3.3 | 9.9 |
| 21:0 i | — | — | — | — | — | — | — | — | — | 2.2 |
| 21:0 ai | — | — | — | — | — | — | — | — | — | 0.5 |
| 21:0 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.4 | 0.3 | 0.2 | 0.2 | — |
| 22:0 | 1.3 | 0.8 | 1.4 | 0.9 | 0.6 | 1.0 | 0.7 | 0.7 | 0.8 | 0.9 |
| 22:3 | — | — | — | — | — | — | — | — | — | 0.5 |
| 22:4 | 0.9 | 1.0 | 0.9 | 1.2 | 1.2 | 1.1 | 1.0 | 0.8 | 1.0 | 0.4 |
| 22:5 | 4.7 | 3.2 | 4.6 | 6.0 | 4.0 | 5.3 | 3.1 | 2.3 | 3.6 | 2.2 |
| 22:6 | 10.4 | 7.6 | 9.1 | 19.9 | 14.8 | 16.1 | 15.5 | 15.0 | 18.9 | 6.7 |

TABLE VI

FATTY ACID UNSATURATION INDEX OF MITOCHONDRIAL MEMBRANE LIPIDS OF GOLDFISH ACCLIMATED TO 5, 20 AND 30°C

| Tissue | Acclimation temperature (°C) | Unsaturation index * | | |
|---|---|---|---|---|
| | | Total | Mono-unsaturated | Poly-unsaturated |
| Red muscle | 5 | 214.8 | 30.3 | 184.5 |
| | 20 | 184.1 | 24.7 | 155.4 |
| | 30 | 201.2 | 33.2 | 168.0 |
| White muscle | 5 | 264.5 | 19.4 | 245.1 |
| | 20 | 226.4 | 21.0 | 205.4 |
| | 30 | 238.5 | 22.7 | 216.8 |
| Liver | 5 | 206.6 | 26.2 | 180.4 |
| | 20 | 198.5 | 24.2 | 174.3 |
| | 30 | 226.7 | 23.1 | 203.6 |
| Food | | 202.8 | 28.8 | 17.40 |

* The unsaturation index is the summation of all double bonds per mol fatty acid, expressed in %.

tion of the 8 major fatty acids found in the membrane lipids (16 : 0, 18 : 0, 18 : 1, 18 : 2, 20 : 1, 20 : 4, 22 : 5, 22 : 6) is clearly different from that of diet lipids. Although the fatty acid composition of mitochondrial membrane lipids are quite different from the diet lipids, it may not be concluded that these diet-lipids were of no importance on the membrane-lipid composition. A more restricted diet, specially with respect to polyunsaturated fatty acids, might have had more pronounced effects.

## Discussion

In contrast with our expectation, isolated mitochondria did not show a simple increase of polyunsaturated fatty acids with decreasing acclimation temperature. (Table VI). Fatty acids of mitochondrial preparations from 5° C-acclimated goldfish are more polyunsaturated than those of 20° C-acclimated goldfish.

However, fatty acids of 30° C-acclimated foldfish are more polyunsaturated than those from 20° C-acclimated, while liver preparations were even more unsaturated than those from 5° C-acclimated foldfish. Comparison of the fatty acid composition is probably not very rewarding since the fatty acid composition does not always change in the same direction as appears from numerous references.

Table VII shows the unsaturation indices from various membrane preparations. Differences between heart and liver mitochondria are demonstrated by Irving and Watson [12] in several fish species, and by Platner et al. [28] in ground squirrels. Liver mitochondria of ground squirrels have very low index values (much lower than rat liver), while squirrel heart has rather high values (much higher than pig heart and comparable to fish-gill and fish-heart mitochondria). It is interesting to see that the unsaturation index of neutral lipids from goldfish mucosa microsomes hardly varies in sharp contrast with the phos-

TABLE VII

FATTY ACID UNSATURATION INDEX OF MITOCHONDRIAL MEMBRANE LIPIDS

A literature survey of the degree of unsaturation of membrane lipids. The unsaturation index is the summation of all double bonds per mol fatty acid expressed in %, and is calculated from given fatty acid compositions. The acclimation temperature or temperature range is given for the poikilothermic animals; the environmental temperature of hibernating *Citellus* is about 5°C.

| References | Species | Preparation | Acclimation temperature (°C) | Unsaturation index |
|---|---|---|---|---|
| Caldwell and Vernberg [22] | Goldfish | Gill mitchondria | 15 | 253 |
| | | | 30 | 233 |
| | Bullhead | Gill mitochondria | 15 | 210 |
| | | | 30 | |
| Richardson and Tappel [41] | Catfish | Liver mitochondria | | 213 |
| | Rat | Liver mitochondria | — | 180 |
| Irving and Watson [12] | Coral trout | Heart mitochondria | 25—30 | 160 |
| | | Liver mitochondria | 25—30 | 145 |
| | Sweet lip | Heart mitochondria | 25—30 | 244 |
| | emperor | Liver mitochondria | 25—30 | 177 |
| | Ring-tailed | Heart mitochondria | 25—30 | 160 |
| | sturgeon | Liver mitochondria | 25—30 | 90 |
| | Mullet | Heart mitochondria | 22—25 | 224 |
| | | Liver mitochondria | 22—25 | 172 |
| Platner et al. [28] | *Citellus* | Heart mitochondria | — | 190 |
| | | Heart mitochondria | Hibernating | 190 |
| | | Liver mitochondria | — | 59 |
| | | Liver mitochondria | Hibernating | 52 |
| Comte et al. [42] | Pig | Heart mitochondria | — | 132 |
| Kemp and Smith [8] | Goldfish | Intestinal mucosa membranes | | |
| | | Phospholipids | 3 | 221 |
| | | Phospholipids | 32 | 151 |
| | | Neutral lipids | 3 | 145 |
| | | Neutral lipids | 32 | 145 |

pholipids [8]. Even within the different classes of phospholipids, as determined in goldfish intestinal-mucosa membranes, different changes in unsaturation and fatty acid composition occur, due to thermal acclimation [29]. In these membranes an increase of the degree of unsaturation of the membrane lipids was found with decrease of acclimation temperature. This effect was also demonstrated by Johnson and Roots [7] in goldfish brain lipids. However, fatty acids from total lipids of whole fish [8] or fish muscle [9] show only very small changes in composition as a result of a changed acclimation temperature. From these results it is very clear that the unsaturated index is probably not a relevant parameter in thermal adaptation studies, since the degree of unsaturation of several membranes in the same organism can vary widely.

Marzuki et al. [30,31] demonstrated that the amount of unsaturated fatty acids not only has some influence on the break-temperature, but is also involved in binding of membrane proteins. They found that in yeast mitochondria, containing less than 34% unsaturated fatty acids, cytochromes were lost, and even below 72% the efficiency of the oxidative phosphorylation was reduced. From these results we could expect a minimal unsaturation index

determined by the protein/lipid ratio. As is shown in Table VII, heart and liver mitochondria have different unsaturation indices which could be related to the large difference of the protein/lipid ratio between those two types of mitochondria. Despite the great difference in unsaturation in heart and liver mitocondria from several fish species, Irving and Watson [12] observed breaks in Arrhenius plots of state III respiration from both preparations almost at the same temperatures. This phenomenon is also demonstrated by our own experiments (Table VI): not only the same break temperatures were found at different unsaturation indices, but also different break-temperatures were found at almost the same unsaturation index. Therefore, we may conclude that there is no correlation between break-temperature and unsaturation level in fish mitochondria.

Ground squirrels are interesting animals to compare with fish, because of their ability to hibernate. Arrhenius plots of state III respiration were found linear when mitochondria were isolated from hibernating ground squirrels and non-linear when isolated from control animals [32]. Raison and Lyons suggested that the linear shape of the Arrhenius plots in the case of the hibernating squirrels was caused by an increased concentration of detergents, cholesterol or unsaturated fatty acids. Plattner et al. [28], analyzed the fatty acid composition of mitochondrial membrane lipids both from hibernating and non-hibernating ground squirrels. As can be seen from Table VII there was no significant change of the unsaturation index. Aloia et al. [33] analyzed the membrane lipid composition of hibernating and non-hibernating ground squirrels. They observed a significant increase of lysophosphatidylcholine in hibernating animals and suggested that increased concentration of lysophosphatidylcholine would prevent a phase transition at low temperatures. However, in liposomes, phase transitions are not influenced by lysophosphatidylcholine concentrations up to 50% (de Gier, J., personal communication). Therefore, it is not certain that: "regulation of phospholipase activity influences the activation energy of mitochondrial respiration by controlled lysophosphatidylcholine production" [33]. Thus, also in squirrel mitochondria changes in the thermotropic behaviour of state III respiration can not be correlated with changes in membrane lipids.

As was stated in the introduction, breaks in Arrhenius plots should occur at higher acclimation temperatures, due to decreasing unsaturation of the membrane lipids, if a correlation exists. This correlation was not found, although a thermally induced shift of the break in Arrhenius plots of mitochondrial respiration could be demonstrated. In Fig. 2 breaks in Arrhenius plots are shown in mitochondria isolated from 3 different tissues. At acclimation temperatures of 30 and 20° C breaks were present in all tissues. At the acclimation temperature of 5° C Arrhenius plots tended toward a more linear shape, especially in red muscle mitochondria. When we started our investigation, breaks in Arrhenius plots in fish mitochondria were not observed. Experiments with fish mitochondria [10,11] revealed linear Arrhenius plots of state III respiration. In 1976 two papers appeared, related to the purpose of our study. Irving and Watson [12] carried out some experiments with tropical fish. They found breaks in Arrhenius plots of succinate oxidase at about 20° C in heart mitochondria as well as in liver mitochondria. Despite different unsaturation levels of membrane fatty acids, the breaks were all at the same temperature. A second

experiment was described by Wodtke [13] with carp muscle mitochondria; he found a significant shift from 15 to 23° C due to a shift of acclimation temperature from 10 to 26° C. This shift has some intriguing aspects because, from the physiological point of view, a transition temperature 5° C above the acclimation temperature has several disadvantages, for at the acclimation temperature the reaction rate will be low and very sensitive to temperature changes. In our studies on goldfish, we found at acclimation temperatures of 20 and 30° C, break-temperatures were always below the acclimation temperature. At the acclimation temperature of 5° C, however, the breaks in the Arrhenius plots occurred between 8 and 12° C. Nevertheless, below the break-temperature the activation energies were rather low.

The oxidative capacity of goldfish tissues showed thermal adaptation. Oxygen consumption of mitochondria and also cytochrome *c* oxidase activity was increased after lowering the environmental temperatures (see Tables II and III). This aspect of temperature adaptation in goldfish has been described by Freed [34], Caldwell [35] and Smit [36]. Part of these compensations can be explained by change in the activity of succinate dehydrogenase, as described by Hazel [37]. He found that the activity can be modulated by lipids. Lipids extracted from cold-acclimated animals reactivate lipid-free succinate dehydrogenase to a higher activity than lipids from warm-acclimated animals. Because membrane-bound enzymes require special lipids [38], modulation of enzyme activity cannot be studied using crude lipid extracts, on which the conclusions of Hazel [37] are based. It may, therefore, be interesting to study the reactivation with different lipid classes, separated from mitochondrial membrane lipids of cold- and warm-acclimated goldfish. It has been demonstrated [39,29] that the composition of the fatty acids esterified at different types of membrane lipids is highly specific. Also the unsaturation of the fatty acids was found dependent on the lipid class. A change of acclimation did not result in a similar change of unsaturation for all lipids [8,29]. Therefore, one can imagine that membrane-bound enzymes can be influenced by just one changed membrane lipid. Thus, in changing the unsaturation of the micro-environment of a protein, the bulk of the lipids in the remainder of the membrane is not necessarily changed too. This idea is based on the annulus model for membrane-bound enzymes, stated by Metcalfe et al. [40]: membrane proteins will select specific lipids from the environment by which a micro-environment can be found.

In conclusion we may state that unless there is no correlation between unsaturation of membrane lipids and breaks in Arrhenius plots of mitochondrial respiration, a regulation by a specific lipid cannot be excluded. This lipid arranged in an annulus around the protein might influence the flexibility of the hydrophobic part of the protein, so the thermotropic behaviour of this complex may be different from the remainder of the membrane lipids.

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