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RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA FROM TISSUES AND ORGANS OF ANTARCTIC FISH*

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Abstract: Research has been carried out on the effect of temperature on the physiological properties of mitochondria isolated from liver, cardiac muscle and encephalon of the Antarctic fish *Notothenia neglecta*. Mitochondria were obtained in a medium containing 0.25 M mannitol, 0.075 M sucrose, 10^{-3} M EDTA, and 10^{-2} M TRIS, in 10^{-3} M phosphate buffer, pH 7.4. All assays were carried out by means of a polarograph assembled with an oxygen electrode possessing a temperature sensor. Respiration rates, oxidative phosphorylation and respiratory control values were assayed in regard to the effect of temperature. All preparations of liver, cardiac muscle and encephalon mitochondria displayed a very efficient respiration mechanism at 1°C. No general pattern for the influence of temperature increase has been found in those preparations. On the other hand, encephalon mitochondria displayed an efficient system for ATP generation by oxidative phosphorylation, but sensitive to temperature increase.

1. Introduction

In 1964, WOHLSCHLAG carried out his classical studies on the concept of metabolic cold adaptation (MCA) of Nototheniid fishes adapted to the McMurdo Sound environment. After WOHLSCHLAG's contribution, assays of the effect of temperature on the rates of oxygen uptake, as a parameter for MCA, have been a frequent subject of investigation.

Research studies on subcellular and metabolic levels, in regard to the effect of temperature, were carried out by BACILA *et al.* (1989) on cardiac muscle sarcosomes isolated from the ice fish *Chaenocephalus aceratus*. It was found that mitochondria physiologic properties such as respiration, oxidative phosphorylation and respiratory control were affected by temperatures higher than that of the natural cold water habitat of this fish. Due to the significance of these results, the present research work

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was designed as a more complete study of the effect of temperature on the respiration rates and oxidative phosphorylation of mitochondria isolated from the liver, cardiac muscle and encephalon of *Notothenia neglecta*. This fish is endemic to the Antarctic seas. It is adapted to the cold water environment of the Antarctic Peninsula, in the Strait of Bransfield, Admiralty Bay, King George Island.

2. Materials and Methods

Sixty-seven specimens of *Notothenia neglecta* (Pisces, Teleostei), were caught by net or hook at Thomas Point and Inlet Martin at Admiralty Bay, King George Island, South Shetlands, from August to October 1994. The specimens were then transported to the Brazilian Antarctic Station and kept in a special aquarium. Blood from each fish was collected in heparin by cardiac puncture and kept for further experiments. After the cardiac puncture, the fish was killed by transfixing the spinal cord with a needle. Liver, cardiac muscle and encephalon were then removed and maintained in cold buffer solution containing 0.25 M mannitol, 0.075 M sucrose, 0.01 M TRIS, 0.01 M phosphate buffer and 0.001 M EDTA, final pH 7.4. This buffer solution was used as an extraction and reaction medium as well. After being cleansed of blood, the organs were homogenised with 3 volumes of the same buffer solution and the whole suspension spun down 15 min at 500× g. The supernatant was then spun down first 15 min at 1500× g and finally 25 min at 6000× g. The sediment from the last step of centrifugation was suspended in 2.0 m*l* reaction medium kept at 0°C in an ice bath and used for further experiments. For the preparation of liver mitochondria, each liver was used separately, while encephalon and cardiac muscle organs of each three animal were pooled together. All steps of dissection and mitochondria preparation were carried out at 0°C. Striated muscle from all fishes was dissected, frozen and transported to our laboratories in Brazil, to be used for biochemical studies.

Assays of respiration and oxidative phosphorylation of *N. neglecta* liver, cardiac muscle and encephalon mitochondria were carried out by polarography by means of an oxygen electrode fitted with a temperature sensor. This model, devised by one of us (P.H.L.), was successfully used by MALUCELLI *et al.* (1995) for studies of viability of the BCG vaccine. Assays of respiration, respiratory control and oxidative phosphorylation were similar to those of CHANCE and WILLIAMS (1955).

The polarograph used was designed after the model of Voss *et al.* (1963), modified and improved by LUCCHIARI and HOSHINO (1980), and LUCCHIARI *et al.* (1984). The reaction chamber (Fig. 1A), made of acrylic, was connected to a water circulating system used to control the temperature. Figure 1B shows the details of the platinum electrode fitted with the temperature sensor. In this instrument a digital thermometer (Fig. 1C) was incorporated into the polarograph described by LUCCHIARI *et al.* (1984). The temperature scale was calibrated using potentiometers P1 and P2.

An oxygen electrode fitted with a sensor for temperature (Fig. 1B) was built according to MALUCELLI *et al.* (1995). A 0.5 mm diameter platinum wire was sealed into a 4 mm diameter soft glass tubing. A 99.95% pure silver ring was sealed around the tip of the tubing in which the platinum wire had been sealed. A diode 1N914 used

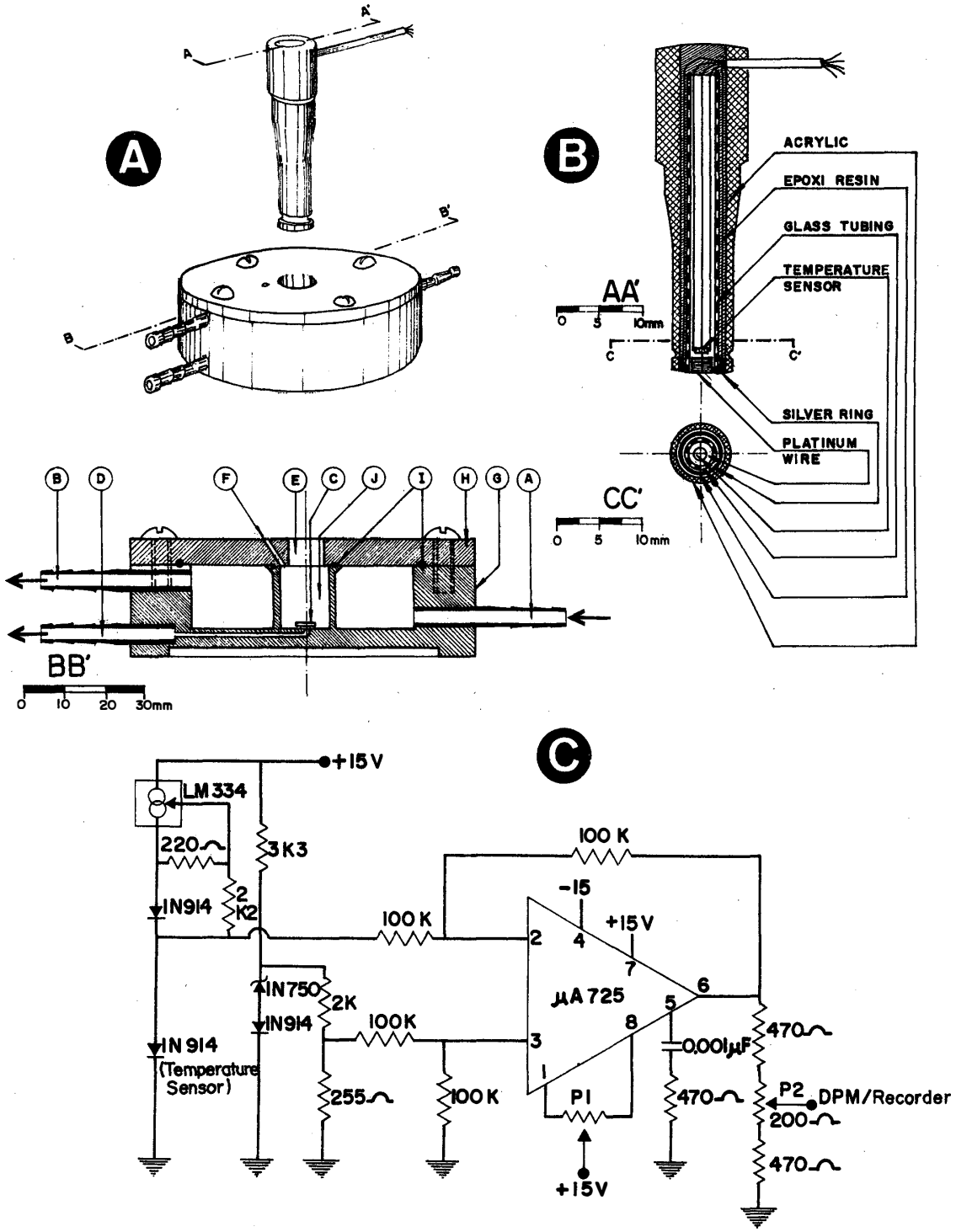

Fig. 1. Schematic view of the reaction chamber (A), the oxygen electrode adapted with a temperature sensor (B), and the electronic circuit of the temperature sensor(C). (A, B and C redrawn from MALUCELLI *et al., 1995) . The reaction chamber (A) is built as follows: A, B = inlet and outlet of circulating water; C, magnetic bar; D, outlet for the cleaning of the reaction chamber by means of a vacuum pump; E, insertion hole for the oxygen electrode and the temperature sensor; F, access hole for the introduction of solutions into the reaction system.; G, main body; H, cover; I, rubber bands for sealing. Outlets A and B are connected with a circulating water bath (sensitivity, 0.1°C); J, Reaction chamber (1.5 cm diameter×2.0 cm height) built in acrylic. For a description of the oxygen electrode, see the text.*

as a temperature sensor was fixed immediately above the silver ring. Copper wires, used to establish outside connections, were soldered to the platinum wire and the silver ring. The whole system was sealed inside acrylic tubing with epoxy resin. After the hardening of the resin, the electrode was carefully polished with #600 water sandpaper and then with flannel. At the end, a dialysis membrane (Sigma's Dialysis "sacks") was used to cover the electrode. It was tied up with nylon thread.

Respiration rates of mitochondria were assayed in a system containing 1.4 m*l* of the reaction buffer (0.25 M mannitol, 0.075 M sucrose, 0.01 M TRIS–HCL, 0.01 M phosphate buffer and 0.001 M EDTA, final pH 7.4). After the equilibrium period that was recorded in a two channel recording device, 0.2 m*l* of mitochondria's suspension was transferred to the reaction chamber and endogenous respiration recorded (Fig. 4). Upon transferring 10 μl of either 0.5 M sodium succinate or α-ketoglutarate pH 7.4 solutions to the reaction chamber, the rate of respiration was assayed in terms of oxygen uptake. For the assay of oxidative phosphorylation and evaluation of the respiratory control, 10 μl of a pH 7.4, 0.05 M ADP solution (final concentration 312 μM) was added to the reaction system. Oxygen uptake corresponding to phase 3 and phase 4 (CHANCE and WILLIAMS, 1955) of the oxidative phosphorylation system, was then recorded (Fig. 4) allowing evaluation of the rate of ATP synthesis and the respiratory control of the preparation. These assays were carried out at temperatures of 1°, 5°, 10°, 22°, and 37°C.

Protein content of the mitochondria preparations assayed by the classical biuret technique was as follows: liver, 59.1±3.4; cardiac muscle, 50.9±13.1; encephalon, 42.3±5.5 mg/m*l*.

To calculate oxygen uptake, the following values of oxygen solubility in water at different temperatures were used: at 1°C, 446 $\mu MO_2/l$; at 5°C, 406 $\mu MO_2/l$; at 10°C, 357 $\mu MO_2/l$; at 22°C, 290 $\mu MO_2/l$; at 37°C, 223 $\mu MO_2/l$. The rate of oxygen uptake was calculated as follows: VO_2 uptake = $(SO_2 \times V_f \times q) / (V_p \times A \times t)$, where SO_2, solubility of O_2 at experimental temperature; V_f, volume of buffered solution in the reaction chamber; q, distance in cm at the ordinate axis of the consumable O_2; V_p,

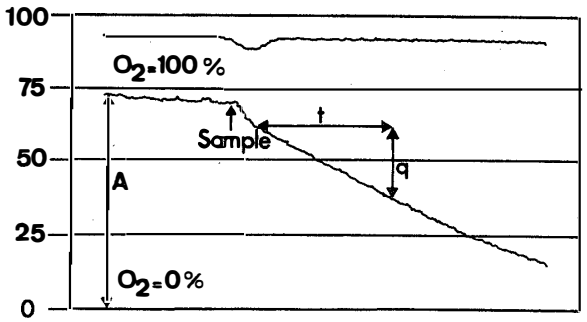

Fig. 2. Graphic model for the polarographic estimation of mitochondria oxygen uptake. The rate of oxygen uptake is calculated as $VO_2 = (SO_2 \times V_f \times q)/(V_p \times A \times t)$. $SO_2 = O_2$ *solubility at experimental temperatures;* V_f, *volume of the buffered solution in the reaction chamber; q, distance in cm at the ordinate axis of* O_2 *uptake;* V_p, *speed of the recording paper; t, distance in cm of the abscissa axis corresponding to the length of time needed for the consumption of* O_2 *(q); A, amplitude of the total oxygen in the system.*

speed of the recording paper; t, distance in cm at abscissa axis, corresponding to the length of time needed for the O_2 consumption (q); A, amplitude of the total oxygen in the system (Fig. 2).

3. Results and Discussion

3.1. Effect of temperature on the rate of mitochondria respiration.

Figure 3 shows the results obtained from the assay of liver, cardiac muscle and encephalon mitochondria respiration performed at different temperatures. The data obtained show a clear effect of the temperature on the rate of mitochondria respiration although a regular pattern of such effect did not appear. In all cases, respiration at 1°C was very efficient. Noteworthy is the observation that respiration of α-ketoglutarate seems to be more sensitive to the effect of temperature than the succinate respiration (Fig. 3). In the case of liver mitochondria, the rate of respiration with succinate as substrate is almost constant in the interval between 1 and 5°C. However, it shows a gradual increase up to 37°C. In this case there is a direct relationship between the temperature rise and the rate of oxygen uptake. Cardiac

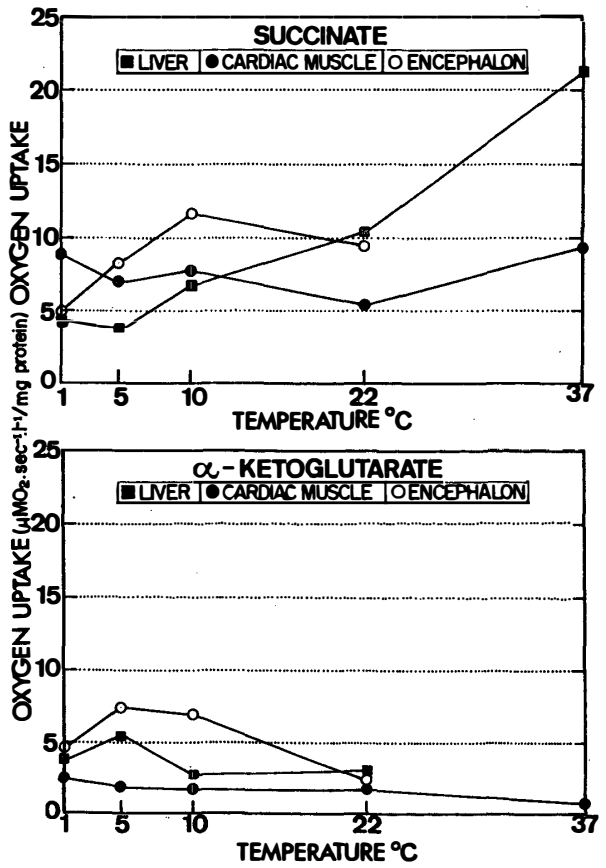

Fig. 3. Respiration rates of Notothenia neglecta*'s liver, cardiac muscle and encephalon mitochondria and the effect of temperature. System.: 1.4 ml of reaction medium containing 0.25 M mannitol, 0.075 M sucrose, 0.01 M Tris, 0.01 M phosphate buffer and 0.001 M EDTA, final pH7.4; 0.23 ml mitochondria suspension; 10 μl of 0.5 M of either succinate or α-ketoglutarate, neutralised to pH 7.4.*

muscle sarcosomes did not behave in the same way: variation of the rates of oxygen uptake at different temperatures is not statistically significant. For the encephalon mitochondria, the rate of oxygen uptake increases linearly from 1 to 10°C. It decreases at higher temperatures. No respiration of the encephalon mitochondria was possible to assay at 37°C.

3.2. *Effect of temperature on the rates of oxidative phosphorylation and respiratory control*

Oxidative phosphorylation of liver, cardiac muscle and encephalon isolated mitochondria was assayed in terms of ADP phosphorylation in systems containing succinate as the respiratory substrate (Table 1). Among all the preparations studied, encephalon mitochondria was found to possess the most efficient system for the generation of ATP by oxidative phospohorylation. Liver mitochondria and cardiac muscle sarcosomes, in spite of being efficient in the process of succinate respiration (Fig. 3) always produced uncoupled preparations that did not respond to ADP in the present experimental conditions.

When oxidative phosphorylation of the encephalon mitochondria was assayed (Table 1) at different temperatures (1–22°C), the records in Fig. 4 were obtained. Oxidative phosphorylation assayed at 1°C showed a very efficient energetic process of tightly coupled mitochondria. However, this mechanism is very sensitive to increasing levels of temperature (Fig. 5). At 5°C the rate of succinate respiration is 2.6 times higher than that at 1°C, but the rate of oxidative phosphorylation is 1.46 times less efficient than at 1°C. At 10°C, the rate of succinate respiration is 3 fold higher than that at 1°C, but the efficiency of oxidative phosphorylation is 1.98 times less efficient than at 1°C. At 22°C, encephalon mitochondria showed less efficiency in both respiration and oxidative phosphorylation.

Determination of respiration rates of heart mitochondria of the ice-fish

Table 1. Effect of temperature on the rates of oxidative phosphorylation and the respiratory control (RC) of liver, cardiac muscle and encephalon mitochondria from Notothenia neglecta. *System: 1.4 ml of reaction medium containing 0.25 M manitol, 0.075 M sucrose, 0.01 M Tris, 0.01 M phosphate buffer and 0.001 M EDTA, final pH 7.4; 0.2 ml mitochondria suspension; 10 µl of 0.5 M of either succinate or α-ketoglutarate; ADP, 312 µM. Values in µM* $O_2 \cdot s^{-1} \cdot l^{-1}$. *Respiratory control is calculated as the rate of respiration between state 3 (ADP) and state 2 (substrate) of respiration. Oxidative phosphorylation is asassayed as the ratio between ADP and oxygen.*

Temperature (°C)	Liver			Cardiac muscle			Encephalon			
	SUC	ADP	RC	SUC	ADP	RC	SUC	ADP	RC	ADP/O
1	3.13	2.56	0.82	12.08	8.00	0.66	3.41	6.83	2.00	1.8
5	3.81	3.57	0.94	—	—	—	8.99	12.29	1.37	2.2
10	5.67	4.67	0.82	—	—	—	10.24	10.35	1.01	1.5
22	7.83	4.24	0.54	17.86	5.48	0.36	8.93	6.48	0.72	2.1
37	23.40	—	—	16.32	1.84	0.11	—	—	—	—

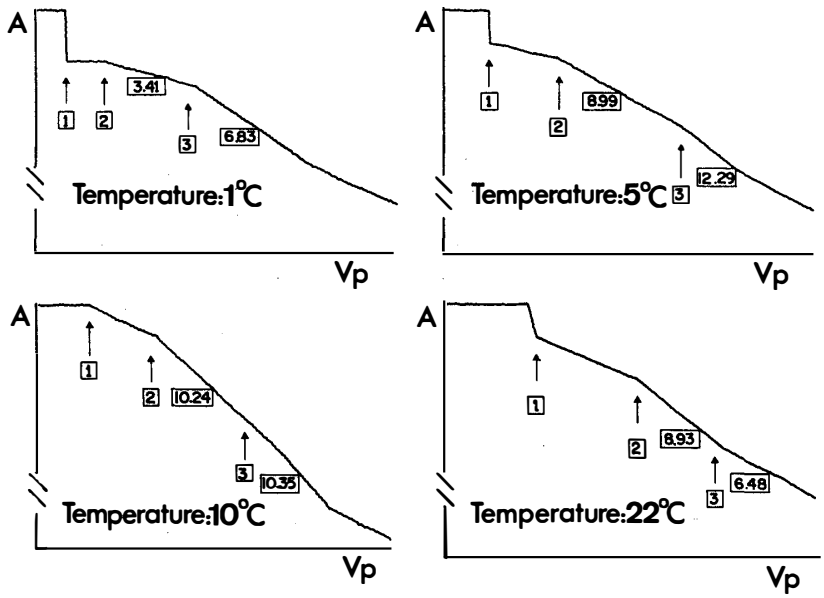

Fig. 4. Polarographic records of the effect of temperature on the respiration and oxidative phosphorylation of Notothenia neglecta*'s encephalon mitochondria. System: 1.4 ml of reaction medium containing 0.25 M mannitol, 0.075 M sucrose, 0.01 M Tris, 0.01 M phosphate buffer, and 0.001 M EDTA, final pH 7.4; 0.2 ml of mitochondria suspension; 10 µl of 0.5 M of succinate; ADP, 312 µM. A= amplitude of the available oxygen in the system; V_p, speed of the recording paper; 1, mitochondria; 2, succinate; 3, ADP.*

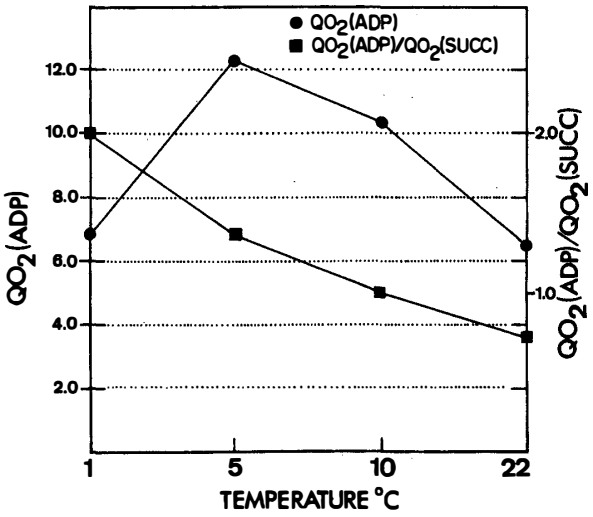

Fig. 5. Respiratory quotient values at the "state 3" of respiration of Notothenia neglecta*'s encephalon mitochondria. System: 1.4 ml of reaction medium containing 0.25 M mannitol, 0.075 M sucrose, 0.01 M Tris, 0.01 M phosphate buffer, and 0.001 M EDTA, final pH 7.4; 0.2 ml of mitochondria suspension; 10 µl of 0.5 M of succinate; ADP, 312 µM.*

Chaenocephalus aceratus by Bacila *et al.* (1989) showed that at temperatures as high as 22.2°C higher rates of oxygen uptake were obtained, the mitochondria still retaining 60% of their metabolic capacity at 2.5°C. At 2.5°C the rate of oxygen uptake for a system containing succinate and α-ketoglutarate was about 61% of the value obtained at 22.2°C. However, the efficiency of the ice-fish heart respiratory chain in liberating energy for the generation of ATP was indicated by the values of the respiratory control obtained at 17.7° and 22.2°C, which were 1.6233 and 1.7343, respectively.

Ever since the work of WOHLSCHLAG (1964) on metabolic cold adaptation (MCA) of Nototheniids, it has been known that if oxygen uptake is a parameter for measurement of the routine metabolic rate, the Nototheniids from McMurdo Sound possess higher rates than those in temperate fishes (EASTMAN, 1993). In these cases the rates found in temperate fishes were extrapolated downward to subzero temperatures.

The data shown in the present paper are an approach to analyze this problem at subcellular and at molecular levels. In regard to the efficiency of oxidative phosphorylation, the encephalon mitochondria seem to possess very efficient energetic machinery displaying a tightly coupled mechanism of generating ATP at 1°C, decreasing its efficiency with increase of temperature. Cardiac muscle sarcosomes and liver mitochondria showed different patterns of responses towards different levels of temperature. On the other hand, these results maybe a new approach towards the concept of MCA for the use of respiration as a measure of temperature compensation. This concept has already been criticized by CLARKE (1983, 1991) on the grounds of respiration being a multiple process as the result of the sum of the rates for a variety of tissues that might represent a cost for the organism. Comparative research work on this very important problem is being carried out in our laboratory with fishes adapted to semitropical and tropical environments. Several metabolic studies with mitochondria preparations from tissues and organs of this fish have already been completed.

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