The Adaptation to Salinity: Response of Fish Gill Mitochondria to Salinity Stress

N. Suresh[1,2] and J. Jayaraman[1]

Abstract

When a freshwater fish, *Tilapia mossambica* (now renamed *Sarotherodon mossambicus*) is exposed to a salinity stress, extensive changes are noted in the properties of the mitochondria isolated from the gill tissue. Our efforts were directed toward ascertaining the response of the gill mitochondria to an ion-osmotic shock to which the organism is exposed. Notable among them are the loss of ability to phosphorylate externally added ADP, decreased $^{45}Ca^{++}$ uptake, lower transhydrogenase levels, and nonresponse to ATP + Mg^{++} for contraction. During this period there is a large influx of calcium into the mitochondria. Continued exposure to the stress situation reverses virtually all the changes to the freshwater levels.

Key Words: Gill mitochondria; salinity adaptation; calcium uptake.

Introduction

To combact a stress situation, the cell has to first mobilize its internal energy resources, before depending on extracellular influences. Mitochondria are the first obvious candidate, and it is therefore to be expected that extensive alterations in the energy-transducing functions of these organelles will take place at the onset of the stress condition. We have demonstrated earlier (Sulochana *et al.*, 1977; Bashyam *et al.*, 1980) that when a freshwater fish *Tilapia mossambica* was exposed to salinity stress, extensive changes were noted in the properties of mitochondria isolated from the muscle tissue. Whereas in the case of the muscle tissue blood serves as an intermediary between the tissue and saline medium, the gill tissue almost remains bathed in

---

[1]Department of Biochemistry, School of Biological Sciences, Madura Kamraj University, Madurai-625021, India.
[2]Present address: Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254.

the medium. It was therefore of obvious interest to investigate the responses of gill mitochondria to the imposed stress. Results of such investigations are reported here.

Materials and Methods

Conditions for collection of fish (*Tilapia mossambica,* now renamed as *Sarotherodon mossambicus*), acclimation to laboratory conditions, and exposure to stress conditions have been described earlier (Sulochana *et al.,* 1977).

Isolation of Mitochondria

At specified time intervals, the fishes were collected from the experimental tanks and decapitated; then the gill tissues were excised out. On the average about six fishes were used per experiment. The excised gills were dropped in cold 0.25 M sucrose, extensively washed to remove any blood, and then blotted out. They were suspended in cold 0.25 M sucrose at 10% w/v concentration and homogenized using a Sorvall tissue homogenizer for 15 sec with intervals of 5 sec each in between. The tissue homogenate was fractionated by differential centrifugation as described earlier (Sulochana *et al.,* 1977).

Analytical Methods

Protein was measured by Lowry's method, using bovine serum albumin as the standard (Lowry *et al.,* 1951). Oxygen uptake rate, respiratory control Index (RCI), and phosphorylation efficiency (ADP/O ratio) were all measured polarographically. The reaction medium and other conditions have been described previously (Honnappa *et al.,* 1975). Density gradient analysis was carried out as described earlier (Bashyam *et al.,* 1980).

Enzyme Assays

Succinate dehydrogenase was assayed by the method of Green *et al.* (1955), NADH oxidase by the method of Mackler (1967), cytochrome *C* oxidase by the procedure of Wharton and Tzagaloff (1967), and ATPase by the method of Tzagaloff (1970).

Energy-Dependent Calcium Uptake

Essentially, the procedure described by Loyter *et al.* (1969) was used with certain minor modifications. The conditions of incubation are described in the legend at appropriate places. After the incubation period, the reaction was stopped by the addition of 0.1 ml of 0.4 M $MgSO_4$ (or 40 mM final

concentration). The contents of the reaction mixture were immediately subjected to centrifugation at 10,000 g for 2 min in a Janetzki model TH 12 centrifuge. The pellets were washed twice in a similar way in cold assay medium, dissolved in minimal amount of 0.1 N NaOH, spotted on Whatman No. 1 filter paper discs, and dried; then the radioactivity was counted in a liquid scintillation counter.

Swelling and Contraction

The medium used contained 0.15 M KCl and 0.1 M Tris-HCl, pH 7.4. Using this medium, zero and 100% transmission were adjusted at 520 nm. Mitochondrial suspension (100–150 μg protein) was added to this so that the optical density was about 0.3 (precalibrated). Immediately after addition of mitochondria and mixing, readings were taken every 15 sec for 2–3 min. After this a mixture of ATP and $MgCl_2$ was added and readings were again taken every 15 sec for an additional 2 min. Details of additions are shown in the respective places. Substrate additions were made where indicated.

Energy-Dependent Transhydrogenase

The method of Kaplan (1967) was used with modifications. The reaction mixture used was the same as the polarographic medium except that it contained, in addition, 0.5 μmol of NADPH and 1 μmol of acetyl pyridine NAD in a total volume of 1 ml. Reaction was initiated by the addition of 100 μg mitochondrial protein, and the optical density was followed at 375 nm. The activity of the enzyme is expressed as the amount of coenzyme reduced per minute per milligram protein.

Estimation of Fatty Acids

The mitochondrial pellet (50 mg protein) was suspended in 3 ml of isolation medium and to this was added 8 ml of 80% methanol, 0.4 ml of 10% ethanolic pyrogallol, and 0.4 ml of 40% ethanolic KOH. The mixture was kept boiling over a water bath for 15 min after which the liberated fatty acids were extracted with ethyl ether. They were converted to methyl esters and analyzed by gas–liquid chromatography as described by Ambudkar et al. (1980). For analyses of phospholipids, the procedure described by Ambudkar et al. (1980) was followed.

Endogenous Levels of Ions

The mitochondrial pellet obtained was washed in 0.25 M sucrose prepared in deionized double distilled water and treated as that of muscle

Table I.   Yield and Respiratory Status of Mitochondria

Parameter	Fish gill	Fish muscle	Rat liver
Yield	3.5 mg/g	1–2 mg/g	10–12 mg/g
Respiratory status:			
Succinate oxidation/			
mg protein (State 4)	21.0 natom O_2/min	5.8 natom O_2/min	14.4 natom O_2/min
ADP/O	1.9	1.8	1.5
RCI for succinate	2.2	2.0	4.0

mitochondria (Bashyam *et al.,* 1980). The ions were estimated using an atomic absorption spectrophotometer (Hitachi–Perkin-Elmer).

Results

Characterization of Fish Gill Mitochondria

In Table I are given the characteristics of mitochondria isolated from gills of freshwater fish. For comparison, the properties of fish muscle mitochondria and rat liver mitochondria are also given.

Effect of Salinity Stress on Mitochondrial Functions

The fish were transferred to 25% seawater or 50% seawater, as the case may be, and on different days, gill tissue mitochondria were isolated and characterized.

Table II.   Respiration on Exposure to Salinity Stress with 25 and 50% Seawater[a]

Days in seawater	State 4 Succinate oxidation (natom O_2/min/mg protein) 25%	50%	ADP/O 25%	50%	RCI 25%	50%
Freshwater	21	21	1.9	1.9	2.2	2.2
1	22	22	1.6	0	2.0	1.0
2	23	22	1.7	0	2.0	1.0
3	22	22	0	0	1.0	1.0
7	22	23	0	0	1.0	1.0
9	24	24	0	0	1.0	1.0
13	22	24	0	0	1.0	1.0
15	21	24	1.9	1.8	2.0	2.0
22	22	24	1.8	1.8	2.0	2.0

[a] The reaction buffer contained 0.25 M sucrose, 0.005 M Tris-HCl, pH 7.4, 0.001 M EDTA, 0.005 M phosphate buffer, pH 7.4, and 0.005 M $MgSO_4$. Mitochondria corresponding to about 3 mg protein were added to the buffer and, after 2 min, 10 mM succinate along with 2 μM rotenone were added. ADP (300 nmol) was added to the reaction mixture to a total volume of 3.5 ml.

(a) *Oxidative Phosphorylation.*   It is seen from the data presented in Table II that the succinate oxidation rate remains unchanged for 22 days irrespective of the magnitude of the stress. But the ability to phosphorylate externally added ADP, measured as ADP/O, is lost on the 3rd day in the case of transfer to 25% seawater and even on the 1st day when the transfer was made to 50% seawater. By about the 15th day, the ADP/O ratio is restored.

(b) *Enzyme Activities.*   A few mitochondrial enzymes like NADH oxidase, cytochrome C oxidase, succinate dehydrogenase, and ATPase were also assayed in intact tissue and isolated mitochondria, and these results are shown in Fig. 1. In all cases, the specific activities remained constant up to 24 days of exposure to salinity. Total activities, however, increase to various degrees, and the significance of this is discussed later.

(c) *Density Gradient Analysis.*   The homogeneity of the mitochondrial preparations from fishes at three stages, namely fresh water, 2 days ("stressed"), and 22 days ("adapted") after exposure to 50% seawater was checked by sucrose density gradient (Fig. 2). Mitochondria from freshwater fish showed a major band at a density corresponding to 1.42 g/cm^3. Mitochondria isolated from fish under stress conditions showed two bands, a minor one corresponding to 1.42 g/cm^3 and a major band at 1.483 g/cm^3. The vast majority of mitochondria from the adapted fishes showed the same density as those from freshwater fish.

(d) *Calcium Uptake.*   The calcium uptake of the different mitochondrial preparations was studied next.

(i) *Kinetics.*   The time course of $^{45}Ca^{++}$ uptake is shown in Fig. 3. The mitochondria obtained from stressed fish take up very little $^{45}Ca^{++}$ up to 22 min, whereas in the mitochondria from freshwater fish there is a linear increase up to 15 min. In the case of mitochondria from adapted fish, there is again uptake, and the kinetics shows more similarity to the kinetics seen with freshwater fish as opposed to stressed fish.

(ii) *Uptake Capacity and Energy Dependence.*   The calcium uptake capacity per milligram of mitochondrial protein by gill tissues isolated at different stages of salinity stress is shown in Fig. 4. When the stress was 25% seawater, there was a 5- to 6-fold decrease in the uptake between 10 and 13 days, which increases to the freshwater levels by 22 days. When the stress was 50% seawater, the decrease of the same extent is observed even on the second day.

The energy dependence of $^{45}Ca^{++}$ uptake by mitochondria at three different stages has also been studied (Table III). As can be seen, the process is not dependent on external ATP or ADP. Omission of succinate causes partial inhibition both in freshwater and adapted mitochondria, whereas no change was seen in the already low uptake level in stressed mitochondria. DNP inhibition was very significant (91% and 84%) in the freshwater and
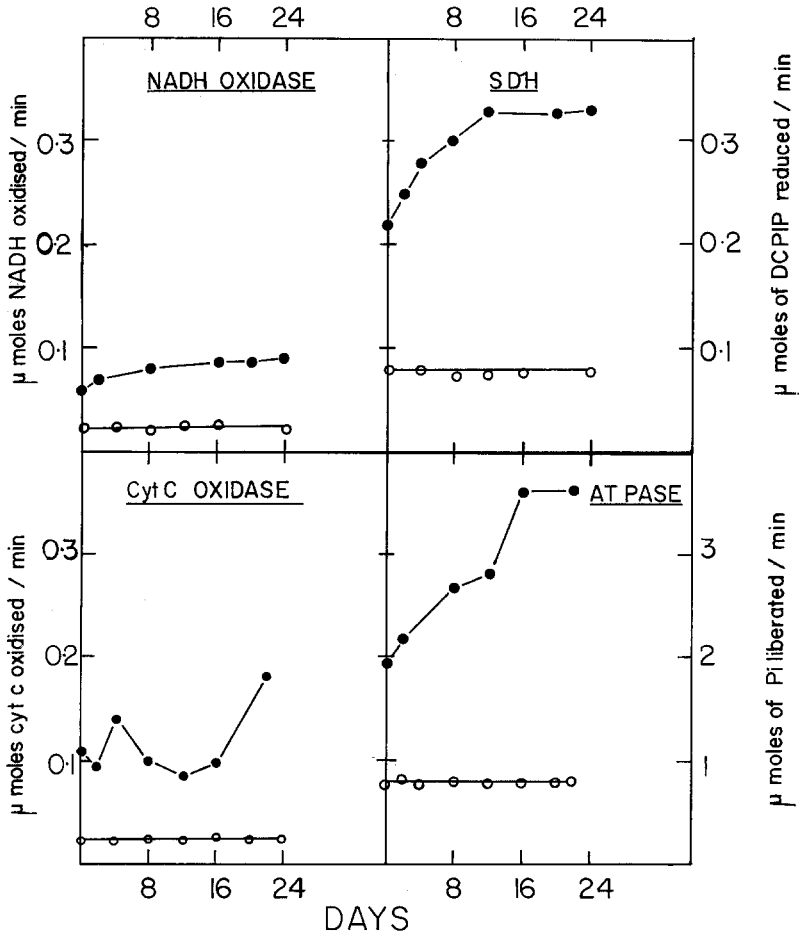
Fig. 1. NaDH oxidase activity during continued exposure to 50% seawater. (●) Total activity (expressed as μmol of NADH oxidized/min/g tissue); (O) specific activity (expressed as μmol of NADH oxidized/min/mg protein). SDH activity during continued exposure to 50% seawater. (●) Total activity (expressed as μmol of DCPIP reduced/min/g tissue); (O) specific activity (expressed as μmol of DCPIP reduced/min/mg protein). Cytochrome C oxidase activity during continued exposure to 50% seawater. (●) Total activity (expressed as μmol of Cytochrome C oxidized/min/g tissue); (O) specific activity (expressed as μmol of Cytochrome C oxidized/min/mg protein). ATPase activity during continued exposure to 50% seawater. (●) Total activity (expressed as μmol of P_i liberated/min/g tissue); (O) specific activity (expressed as μmol of P_i liberated/min/mg protein).
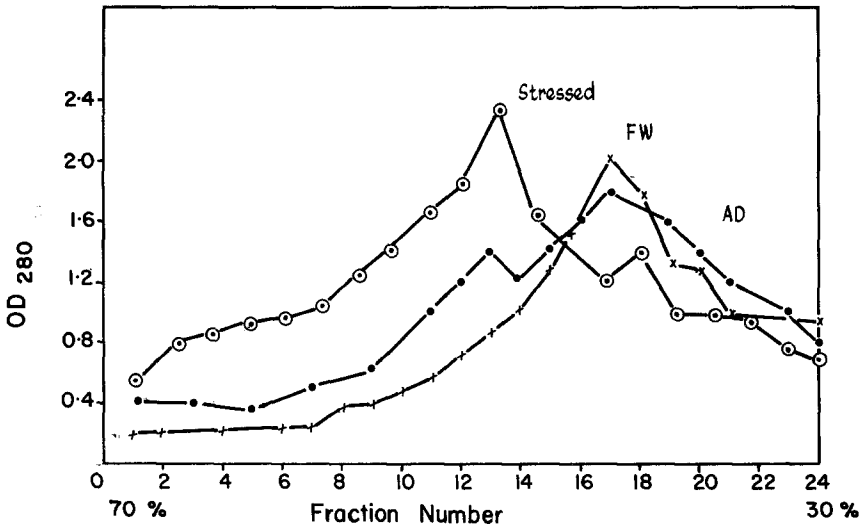
Fig. 2. Density gradient analysis of mitochondria layered on sucrose gradient (70–30%). (×) Freshwater mitochondria; (⊙) stressed mitochondria (2 days of exposure of the fish to 50% seawater); (●) adapted mitochondria. FW, freshwater; AD, adapted.
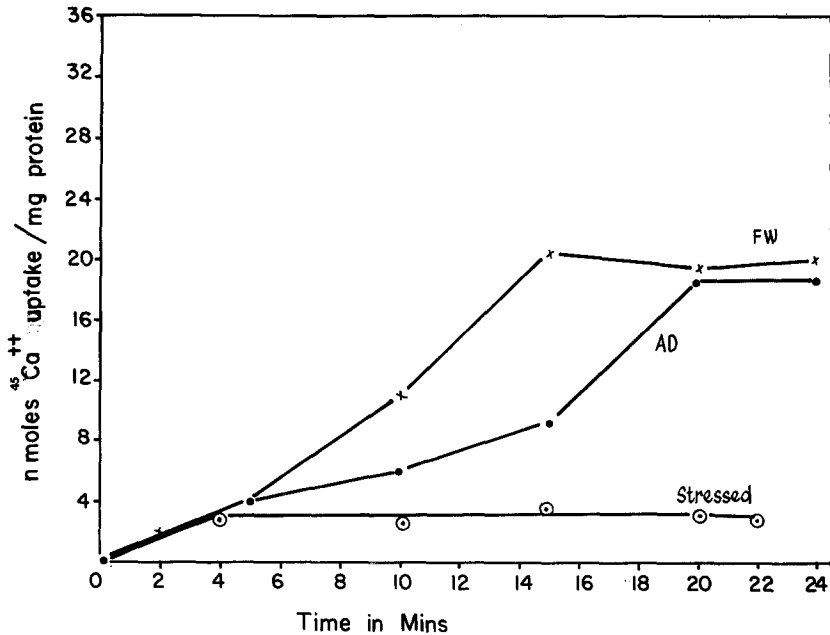

Fig. 3. Time course of ^{45}Ca uptake in freshwater, stressed, and adapted mitochondria (nmoles ^{45}Ca^{++}/mg protein). (×) Freshwater mitochondria; (⊙) stressed mitochondria (2 days of exposure of the fish to 50% seawater); (●) adapted mitochondria. The assay system contained in 1 ml reaction mixture 0.15 M KCl, 0.01 M Tris-SO$_4$, pH 7.4, 0.05 M phosphate buffer, pH 7.4, 10 mM succinate, 7.5 mM ATP, and 1–2 mg mitochondrial protein. ^{45}CaCl$_2$ (200 nmol) was added to start the reaction. The reaction was stopped by the addition of 0.1 ml of 0.4 M MgSO$_4$ after 15 min of incubation with shaking.
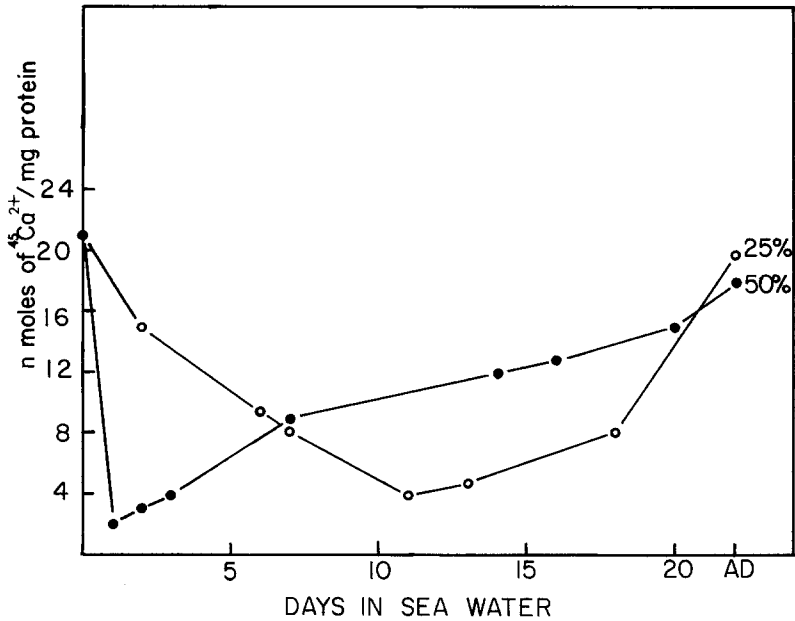
Fig. 4. ^{45}Ca uptake in mitochondria on different days of exposure to 50 and 25% seawater (nmol of $^{45}Ca^{++}$/mg protein). (O) 25% seawater; (●) 50% seawater. The assay system contained in 1 ml reaction mixture 0.15 M KCl, 0.01 M Tris-SO$_4$, pH 7.4, 0.05 M phosphate buffer, pH 7.4, 10 mM succinate, and 7.5 mM ATP. 1–2 mg mitochondrial protein was used per assay. $^{45}CaCl_2$ (200 nmol) was added to start the reaction. The reaction was stopped by the addition of 0.1 ml of 0.4 M MgSO$_4$ after 15 min of incubation with shaking.

Table III. $^{45}Ca^{++}$ Uptake[a]

	Freshwater		Stressed		Adapted	
Condition	nmol/mg	% Inhibition	nmol/mg	% Inhibition	nmol/mg	% Inhibition
+ Succinate + ATP	19.0	—	3.0	—	23.4	—
– ATP	18.3	4	3.3	—	23.6	—
– Succinate	13.0	32	3.1	—	20.0	15
– ATP + ADP	18.9	—	4.6	—	19.4	17
+ DNP	1.7	91	1.5	50	3.8	84

[a] The assay system contained in 1 ml reaction mixture 0.15 M KCl, 0.01 M Tris-SO$_4$, pH 7.4, 0.05 M phosphate buffer, pH 7.4, 10 mM succinate, 7.5 mM ATP, and 1–2 mg mitochondrial protein. $^{45}Ca^{++}$ was added to start the reaction. The reaction was stopped by the addition of 0.1 ml of 0.4 M MgSO$_4$. Where indicated, 3 μM ADP and 1 mM DNP were used. Input of $^{45}CaCl_2$ 200 nmol/ml. Incubation time 15 min.

Table IV. Swelling and Contraction[a]

Conditions	Freshwater (Δ OD/min)	Stressed (Δ OD/min)	Adapted (Δ OD/min)
Endogenous swelling rate	−0.010	−0.010	−0.010
Swelling rate with succi- nate	−0.010	−0.010	−0.010
ATP + Mg⁺⁺ addition after succinate	+0.015	−0.015	+0.015

[a]The medium contained 0.15 M KCl and 0.1 M Tris-HCl, pH 7.4. Mitochondrial suspension (100–150 μg protein) was added to this medium, and after mixing, 25 mM succinate was added and the optical density at 520 nm measured every 15 sec for 2–3 min. A mixture of 5 mM ATP and 5 mM MgCl₂ was added at the time indicated on Fig. 5, and the optical density was measured every 15 sec for another 3 min.

adapted mitochondria, but there was only a 50% inhibition in stressed mitochondria.

Swelling and Contraction

The rate of swelling remained more or less the same in all three cases (Table IV and Fig. 5). Addition of succinate did not show any effect. But on addition of ATP + Mg⁺⁺, contraction was observed only in freshwater and adapted mitochondria, but the stressed mitochondria continue to swell at a slightly increased rate (Table IV and Fig. 5).

Transhydrogenase

The results of assay of transhydrogenase activities in freshwater and stressed mitochondria are given in Table V (unfortunately, the adapted mitochondria were not assayed). Substrate additions enhance the endogenous activity of freshwater mitochondria almost twofold and the activity is sensitive to azide and DNP. The stressed mitochondria has about 20% lower endogenous activity and, although stimulated by substrates, the extent is not as high. But importantly, azide did not inhibit the activity whereas DNP did in stressed mitochondria.

Chemical Characterization

A series of experiments was carried out to determine the chemical composition of these mitochondria isolated at different stages of stress.

(a) Nucleic Acids. The variations of mitochondrial DNA and RNA levels during various days of stress in 25% seawater have already been published (Meenakshi et al., 1979). These results indicated some fluctuations
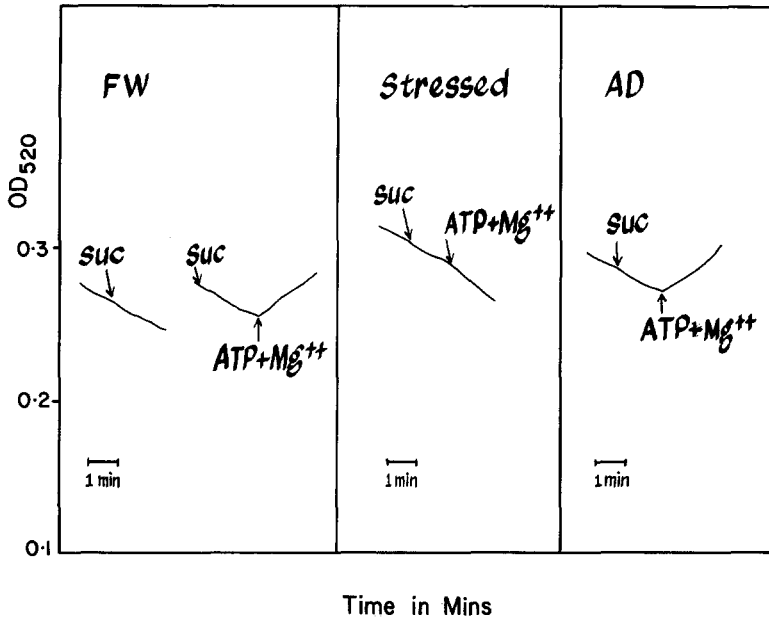
Fig. 5. Swelling and contraction pattern of isolated freshwater, stressed (2 days of exposure of the fish to 50% seawater), and adapted mitochondria. The medium contained 0.15 M KCl and 0.1 M Tris-HCl, pH 7.4. Mitochondrial suspension (100–150 μg protein) was added to this medium and, after mixing, 25 mM succinate was added and the optical density at 520 nm was measured every 15 sec for 2 to 3 min. A mixture of 5 mM ATP and 5 mM MgCl₂ was added at the time indicated, and the optical density was measured every 15 sec for another 3 min.

Table V.   Transhydrogenase[a]

Condition	Freshwater (μmol/min/mg)	Stressed (μmol/min/mg)
Endogenous rate	0.0098	0.0078
+ Succinate + rotenone	0.0178	0.0105
+ ATP	0.0143	0.0105
+ ATP + azide	Nil	0.0105
+ Glutamate + malate	0.0214	0.0209
+ Glutamate + malate + azide	Nil	0.0105
+ TMPD + ascorbate + antimycin	0.0160	0.0105
+ TMPD + ascorbate + antimycin + azide	Nil	0.0105
+ Succinate + rotenone + DNP	Nil	Nil

[a]Succinate (10 mM), glutamate (10 mM), malate (5 mM), TMPD (1 mM), ascorbate (10 mM), rotenone (2 μM), antimycin (1 μM), azide (10 mM), ATP (5 mM), and DNP (1 mM) were added where indicated. The reaction medium was the same buffer used for oxygen uptake studies except that it contained, in addition, 0.5 μmol of NADPH and 1 μmol of acetylpyridine NAD in a total volume of 1 ml. The reaction was initiated by the addition of 100 μg mitochondrial protein and the optical density was monitored at 375 nm. The activity was expressed as coenzyme reduced per min per mg protein.

Table VI. Mitochondrial Protein–Lipid Levels[a]

Days of stress (50% seawater)	Protein (mg/g tissue)	Total lipid/ protein ratio	Phospholipids (μmol P$_i$/g tissue)					
			Total	LPE	PS	PE	PG	CL
0	3.26	30/70	0.913	0.065	0.130	0.490	0.163	0.065
2 (stressed)	3.66	27/73	0.952	0.073	0.110	0.550	0.146	0.073
4	4.00	—	—	—	—	—	—	—
8	4.16	—	—	—	—	—	—	—
16	4.60	—	—	—	—	—	—	—
22 (adapted)	4.70	24/76	1.080	n.d.	0.140	0.705	0.140	0.094

[a]LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin. n.d., not determined.

in the initial stages but after about five or six days showed a steady and significant increase.

(b) *Proteins.* Barring insignificant initial fluctuations after transfer to stress conditions, there appeared to be an increase in the mitochondrial protein content per gram tissue (from about 3–4.8 mg) after continued exposure for 15 days or more (Table VI; see also Meenakshi *et al.*, 1979).

(c) *Total Lipids.* Total lipids-to-protein ratio has also been included in Table VI. There is a slight decrease in the ratio.

(d) *Phospholipids.* The data on phospholipids (Table VI) shows an increase (1.2-fold) during the entire period. Since the extent of increase is slightly lower than that of the protein (1.44-fold), this accounts for the decrease observed in the lipid/protein ratio. Two significant points to be noted are that both PE considered as "structural" phospholipid and CL considered as "functional" phospholipid show an increase.

(e) *Fatty Acids.* The total fatty acid analysis data given in Table VII show that there is no qualitative variation. Quantitative variations are, however, seen. There is an initial decrease from 75 to 40 μmol/g tissue in the first 2 days, which recovers to 50 mmol/g tissue by the 22nd day. Correlation of these values with the data on phospholipids is difficult at present, since total fatty acids have only been analyzed.

Ionic Levels

Endogenous levels of sodium, potassium, magnesium, and calcium have also been measured (Fig. 6). It can be seen that an immediate effect of the stress seems to be a considerable increase in the levels of Ca^{++}, Mg^{++}, and K^+, whereas Na^+ shows a twofold decrease. As in all other cases, continued exposure to stress normalizes the levels by about 15 days.

Table VII. Fatty Acid Analysis

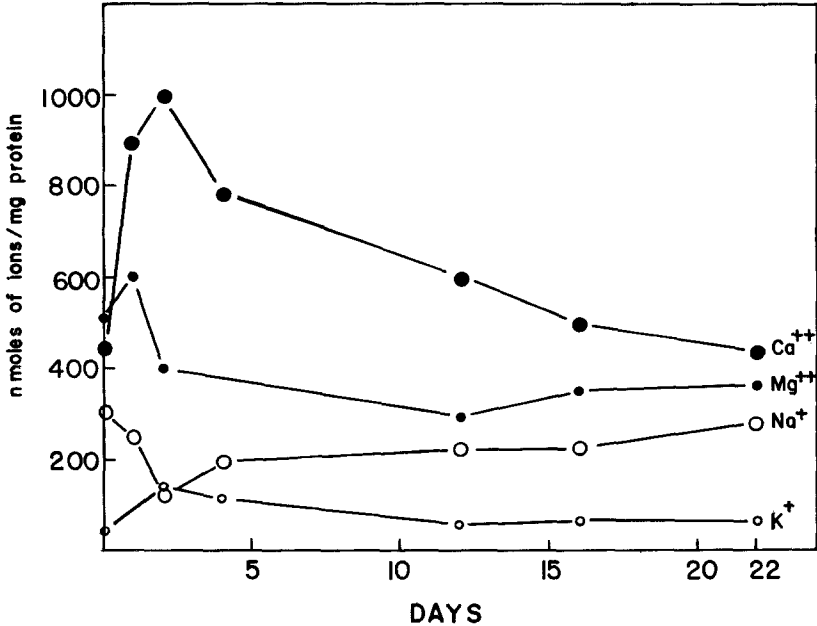| Days	$C_{18:1}$ Oleate	C_{18} Stearate	$C_{16:1}$ Palmitoleate	C_{16} Palmitate	C_{14} Myristate	C_{12} Laurate	Total		UFA/SFA
							μmol/mg protein	μmol/g tissue	
Freshwater	3.5	5.7	2.6	7.0	1.7	2.4	22.9	74.65	0.36
1	2.0	4.3	2.5	5.7	2.2	2.4	19.1	66.90	0.31
2	1.2	3.0	0.7	4.2	0.7	1.0	10.8	39.53	0.21
8	2.0	2.8	0.9	2.5	0.5	0.7	9.4	39.10	0.45
Adapted	2.4	3.6	0.8	3.1	0.5	0.4	10.8	50.16	0.42

Fig. 6. Endogenous levels of ions in the mitochondria on different days of exposure to 50% seawater (nmol of ions/mg protein). (●) Ca^{++}; (○) Na^+; (●) Mg^{++}; (○) K^+.

Discussion

In this communication, we have tried to characterize the changes taking place in the mitochondria of gill tissue of a freshwater fish when it is given an ionosmotic shock. Preferring an overview at this stage, rather than a lengthy discussion of each result presented, we propose the following sequence of events.

(a) Immediately on exposure to the stress situation, changes take place in mitochondrial functions. Striking among them are the loss of ability to phosphorylate externally added ADP, the decreased ^{45}Ca uptake capacity, the lower transhydrogenase levels, and the nonresponse to ATP + Mg^{++} for contraction [data presented here and the enhanced *in vitro* incorporation of amino acids into proteins (unpublished results)]. Mitochondria also tend to remain in a swollen state (Fig. 5). Electron-microscopic pictures tend to confirm this (data not presented). Interestingly, during all these changes the succinate oxidation (State 4) rate remains almost constant, suggesting a constant generation of membrane potential.

(b) Concomitant with these changes are some structural alterations, namely appearance of five new species of proteins and decrease in membrane fluidity (see accompanying paper)

(c) Analyses also reveal that during this period, there is a large influx of calcium into the mitochondria (ionic level measurements and higher buoyant density). Ca^{++} is known to induce several of the changes discussed in (a). Calcium loading has been shown to uncouple oxidative phosphorylation (Villalobo and Lehninger, 1980), and calcium-loaded mitochondria show no stimulation of oxygen uptake by ADP. Similarly, high endogenous levels of calcium cause inhibition of energy-dependent transhydrogenase (Andreoli *et al.*, 1964) and also swelling of the mitochondria (Brierley, 1976). The inability of ATP + Mg^{++} to induce contraction of the swollen mitochondria could be ascribed to the possibility of calcium competing with ATP and Mg^{++} for the binding sites in mitochondria. However, we are not in a position at the moment to dwell on the cause-and-effect relationship governing the aforementioned phenomena.

(d) Continued exposure to the stress situation reverses virtually all the changes to the freshwater levels.

(e) It is further clear from the data [increase in total activities of enzymes, protein content, and cardiolipin content (this paper) and increase in DNA, RNA, and heme levels (Meenakshi *et al.*, 1979)] that during this phase of adaptation mitochondriogenesis occurs.

From what has been said above, two questions arise: (1) In what way does mitochondriogenesis help the fish to adapt to the stress? and (2) What are the triggers for mitochondriogenesis? Some of these aspects will be addressed in our subsequent communications.

Acknowledgments

References

Ambudkar, S. V., Khan, S. L., and Jayaraman, J. (1980). Phospholipid metabolism in *E. coli* during growth in high glucose medium, *Indian J Biochem. Biophys.* **17**, 361–365.

Andreoli, T. E., Pharo, R. L., and Sanadi, D. R. (1964). On the mechanism of oxidative phosphorylation: further evidence for transhydrogenase reaction in submitochondrial particles, *Biochim. Biophys. Acta* **90**, 16–23.

Bashyam, I., Suresh, N., and Jayaraman, J. (1980). Adaptation to salinity by fish: alterations in energy transducing status of muscle mitochondria, *J. Biosci.* **2**, 87–98.

Brierley, G. P. (1976). The uptake and extrusion of monovalent cations by isolated heart mitochondria, *Mol. Cell. Biochem.* **10**, 41–62.

Green, D. E., Mil, S., and Kohout, P. M. (1955). Studies on the terminal electron transport system, *J. Biol. Chem.* **217**, 551–567.

Honnappa, G. V., Sulochana, H., and Jayaraman, J. (1975). Respiratory and calcium accumulating properties of muscle mitochondria of some aquatic arthropods, *J. Bioenerg.* **7**, 149–159.

Kaplan, N. O. (1967). *Methods Enzymol.* **10**, 317.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with folin phenol reagent, *J. Biol. Chem.* **193**, 265–275.

Loyter, A., Christiansen, R. O., Steensland, H., Saltzgager, J., and Racker, E. (1969). Energy-linked ion translocation in submitochondrial particles, *J. Biol. Chem.* **244**, 4422–4427.

Mackler, B. (1967). *Methods Enzymol.* **10**, 261.

Meenakshi, S., Rajamanickam, C., and Jayaraman, J. (1979). Adaptation to salinity by fish: macromolecular changes in mitochondria and microsomes of the gill, *J. Biosci.* **1**, 427–432.

Sulochana, H. R., Bashyam, I., Suresh, N., and Jayaraman, J. (1977). Physiological correlates of calcium-accumulating properties of mitochondria: fish muscle mitochondria, *J. Bioenerg. Biomembr.* **9**, 337–348.

Tzagoloff, A. (1970). Assembly of the mitochondrial membrane system, *J. Biol. Chem.* **245**, 1545–1551.

Villalobo, A., and Lehninger, A. L. (1980). Inhibition of oxidative phosphorylation in ascites tumor mitochondria and cells by intramitochondrial calcium, *J. Biol. Chem.* **255**, 2457–2564.

Warton, D. C., and Tzagoloff, A. (1967). *Methods Enzymol.* **10**, 245.