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Physiological Correlates of Calcium-Accumulating Properties of Mitochondria: Fish-Muscle Mitochondria

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Abstract

The mitochondria isolated from the muscles of fish acclimated to grow in different salinities have been studied with reference to their Ca^{2+} uptake capacity and compared to those isolated from fresh-water fish muscle. The results show a drastic response by the mitochondria with reference to their Ca^{2+} uptake function soon after exposure to the stress. Evidence is also presented to suggest an alteration in conformation. This perturbation appears to be the initial response to the stress since the normal state (as that of the fresh-water fish) is restored in course of time. Further, so far there is no indication that the electron transport function and ATP production are affected by the ionic stress conditions. This would support the physiological relevance of the mitochondrial capacity for Ca^{2+} uptake.

Introduction

The capacity of mitochondria to sequester calcium ions in an energy-dependent fashion is well established. Would this mean that the organelle functions as an intracellular ionoregulator? This question has been occupying our attention for the past few years. Among the various avenues

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open to answer this, we have chosen the broader approach of trying to find out whether this Ca^{2+} uptake capacity has any physiological correlates. A survey of mitochondria isolated from muscle tissues of some arthropods [1], showed correlation between the Ca^{2+} uptake capacity and the calcium need of the organism. A recent study, unpublished‡, on the mitochondria isolated from the mantle tissues of snail and bivalve, also tended to confirm the usefulness of this approach.

Another useful approach would be to study the response of the mitochondria, from a tissue, when the organism is subject to stress conditions, particularly ionic stress. The logic of this rather open-ended series of experiments was that the mitochondria, if it has any role in ionoregulation, should respond to external ionic stress.

In this paper, we present our data on the positive response of mitochondria of the muscle tissues of the fresh-water fish *Tilapia mossambica* when the organism was exposed to ionic stress in terms of salinity. This fish, an euryhaline teleost, has been used because it can adapt itself to different salinities ranging from fresh water to full-strength sea water. The muscle mitochondria of the fish does respond rather drastically, both qualitatively and quantitatively, when the organism as a whole is subjected to ionic stress. Evidence is presented to show alterations, both in function and also in conformation.

Materials and Methods

The Maintenance of the Fish

The fish *Tilapia mossambica*, used in these experiments were collected from the local ponds and selected for uniform size (about 12 cm long, average weight 5 g). The selected fish were kept in aquaria with good aeration and were fed with minced meat to satiation once daily. Tap water was used to fill the aquaria tanks and renewed every alternate day. The fish were left undisturbed for 4–5 days after bringing them from the pond before use for any experiments. These were controls or fresh-water group.

Acclimation to Various Salinities

Stepwise Exposure. A set of fishes were transferred to a tank containing sea water diluted to 25% with tap water and left for 1 week for acclimation and were transferred on the eighth day to a tank containing sea water diluted to 50% with tap water, and left for another 7 days. Above this level, gradual acclimation was adopted by the natural evaporation of water due to rapid

‡ This work has since been published by Hannah R. Sulochana, G. V. Honnappa, and J. Javaraman, *Indian J. Exp. Biol.*, **15** (1977), 1102–1104.

aeration. As the concentration reached around 70% sea water, they were transferred to 75% sea water and further on to 100% sea water after another week. The salinity at each stage was measured and maintained by using YSI Salinometer.

Direct Exposure. The fishes were introduced to the required salinity adjusted by diluting the sea water with fresh water, and maintained in this condition for the required period of time.

Isolation of Muscle Mitochondria

About 6–8 fishes were killed for each experiment. They were decapitated, the skin was peeled off, and skeletal muscle was collected by scraping. The yield was about 0.5–0.6 g wet weight per fish. The muscle tissue was minced well, homogenized, and mitochondria isolated as described earlier [1]. The isolation medium used was 0.1 M KCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, and 5 mM $MgSO_4$. This media was chosen after a number of trials and was found to give optimal and reproducible results. The final mitochondrial pellet was washed twice with cold EDTA-free isolation medium and suspended in appropriate reaction media at a concentration of 15 mg protein/ml. All the operations were carried out at temperatures of 0–4°C.

Analytical Methods

Protein was measured by Lowry's method [2]. Oxygen uptake was measured polarographically using a Clark-type oxygen electrode. Methods for study of Ca^{2+} uptake, both energy-dependent and energy-independent, have been described in detail in the earlier paper [1].

The mitochondria after incubation with radioactive ^{45}Ca under appropriate conditions were collected in Millipore filters in most of the experiments. In other experiments, the incubated samples were quenched with 40 mM $MgSO_4$, quickly centrifuged in a Janetski TH-12 model (10,000 *g* for 2 min) the pellets were washed once with buffer and resedimented by centrifuging for another 2 minutes. The pellets were transferred to planchets with the aid of water and radioactivity determined with a GM counter (efficiency 1.8%). Control experiments showed no differences between filtration and centrifugation techniques. The latter method was preferred since 18 samples could be handled simultaneously.

Chemicals

Analytical grade reagents were used throughout. $^{45}CaCl_2$ was purchased from the Bhabha Atomic Energy Research Centre, Bombay. ATP was from

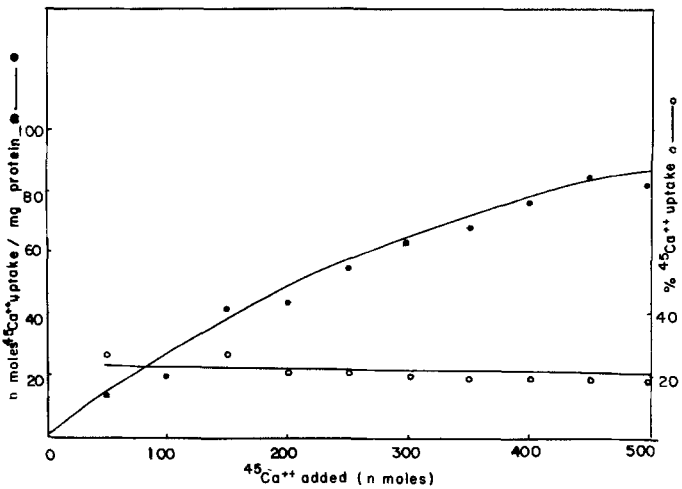

Figure 1. Energy-linked calcium uptake as a function of calcium concentration. Assay system contained in 1 ml reaction mixture (0.15 M KCl; 0.01 M Tris-SO_4, pH 7.4; 0.05 M phosphate buffer, pH 7.4; 5 mM succinate, 7.5 mM ATP, and 1–2 mg mitochondrial protein. $^{45}Ca^{2+}$ was added in the mentioned concentrations.

Centron Laboratory, Bombay, and ADP was from Sigma Chemical Company, USA.

Results

Mitochondria from the Muscles of Fresh-Water Fish

Mitochondria were isolated from the muscles of fresh-water fish and characterized. As there was a high degree of reproducibility from batch to batch, only representative values are given.

Respiratory Capacity. A number of substrates were tried and only succinate gave maximum values. The state III oxidation rate was 9.6 nmol O_2 used/min/mg mitochondrial protein. The respiratory control index was generally on the lower side (2.0) but the ADP/O ratio was 1.6. In all further experiments, only those preparations which gave these values or better were used.

Energy-Dependent Ca^{2+} Uptake. These mitochondria were capable of sequestering Ca^{2+} ions in an energy-dependent manner. Figure 1 gives the data on the amount of $^{45}Ca^{2+}$ taken up as a function of $^{45}Ca^{2+}$ added in the medium. The saturation kinetics are not observed. For the range of Ca^{2+} added, 50–500 nmol, the percentage uptake remained constant, around 20%. This indeed is peculiar behavior, but one possible explanation could

be unspecific permeation in the extramitochondrial space. Although this parameter is being currently looked into along with our studies on the swelling–contraction properties of these mitochondria, we tend to discount the possibility based on the results in Table I, where DNP addition is shown to cause a 80–90% inhibition of the uptake.

The kinetics of $^{45}Ca^{2+}$ uptake has been studied (Fig. 2). There was a steady increase till about 20 min and thereafter it plateaus out. The same pattern is obtained whether one uses a low level of Ca^{2+} (120 nmol) or a high level (4 μmol). That this uptake is energy-linked is shown by the data in Table I, where it is seen that either the omission of substrate, ATP or addition of DNP decreases the uptake drastically. This uptake was inhibited by antimycin and rotenone (data not given).

The activation energy for this process has also been studied by means of Arrhenius plots (Fig. 3). A biphasic curve is obtained, with a transition point at 15°C. At normal temperatures of fish growth and mitochondrial study (30°C), the activation energy was 12.1 Kcal/mole.

Energy-Independent Ca^{2+} Uptake. The ability of these mitochondria to bind Ca^{2+} in presence of the electron transport process inhibitors, antimycin and rotenone, has also been studied. The results are represented in the conventional way as Scatchard plots in Fig. 4a. A monophasic line is obtained, showing only one type of binding. The number of affinity sites

TABLE I. Energy dependency of calcium uptake function during long-term exposure to salinity as given in Figure 6

Assay	Fresh water	25% Sea water		50% Sea water	
		day 8	day 13	day 1	day 13
I[a]					
Complete	32.0	6.8	11.1	3.3	22.0
– ATP	11.5	2.1	2.2	2.9	17.0
+ DNP	3.0	2.3	2.4	2.2	3.8
– Succinate	12.0	2.5	2.8	2.6	15.0
II[b]					
Complete	300	47.6	70.1	75	157
– ATP	65	35.5	43.6	66.5	121
+ DNP	60	40.8	57.5	47.6	51.3

[a] 164 nmol $^{45}CaCl_2$.
[b] 4164 nmol $^{45}CaCl_2$.

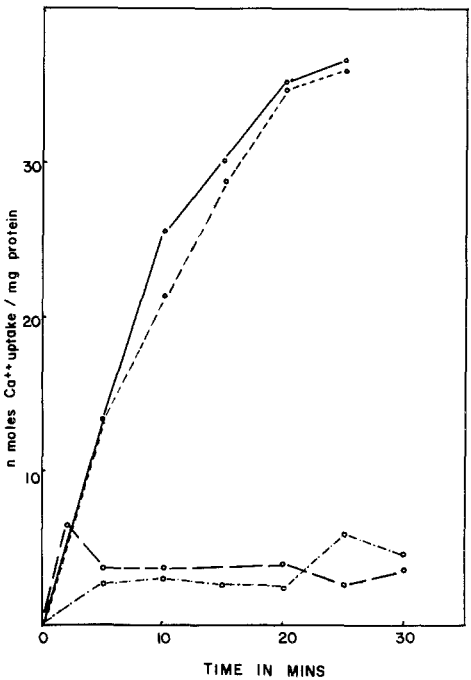

Figure 2. Energy-linked calcium uptake as a function of time. (O————O) Fresh water (control value); (O–·–·–O) seventh day of exposure to 25% sea water; (O—— ——O) eighth day of exposure to 25% sea water; (O– – – – –O) twenty-first day of exposure to 25% sea water. Assay system is complete, as mentioned in Figure 1.

(8.0 nmol/mg protein) and affinity constant (62.5 μM), calculated according to the method of Reynfarje and Lehninger [13], would categorize them as belonging to the low affinity, Table II.

TABLE II. Energy-independent calcium binding sites and their affinity constants in the muscle mitochondria of *Tilapia mossambica* acclimated to different salinities during stepwise acclimation

	Affinity sites		K_d (μM)[a]	
salinity level	High	Low	High	Low
Fresh water				
7 days	—	8	—	62.5
25% Sea water				
7 days	0.5	9	1.8	250
50% Sea water	—	10	—	22

[a] K_d, the binding affinity constant, is $1/K$ where K is the velocity constant.

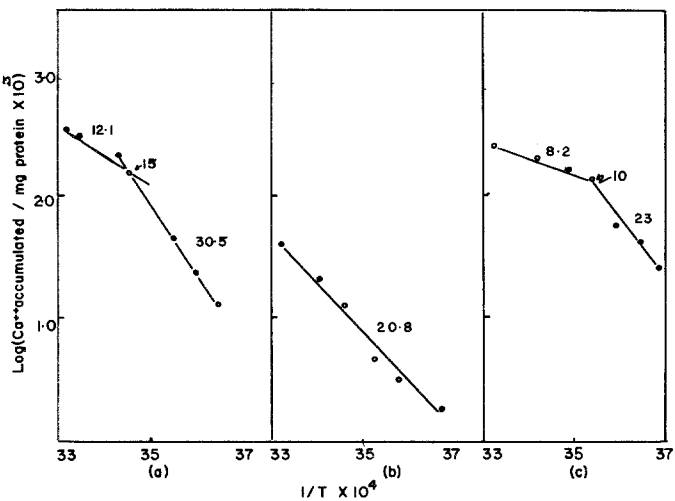

Figure 3. Arrhenius plots of energy-dependent calcium uptake. The experimental conditions are described in Figure 1. (a) Fresh water; (b) eighth day of exposure to 25% sea water; (c) eighth day after transfer to 50% sea water from 25% sea water.

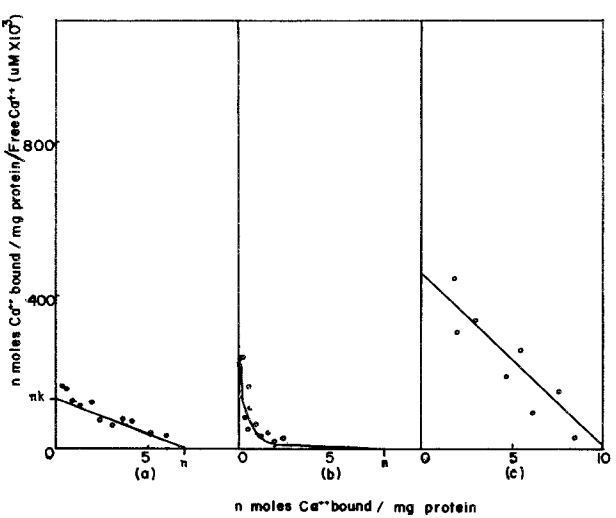

Figure 4. Scatchard plots of the energy-independent membrane binding of calcium. (a) Fresh water; (b) eighth day of exposure to 25% sea water; (c) eighth day after transfer to 50% sea water from 25% sea water. The number of binding sites and affinity constants are derived from the nK on the ordinate and n on the abscissa (reference 3; Table II).

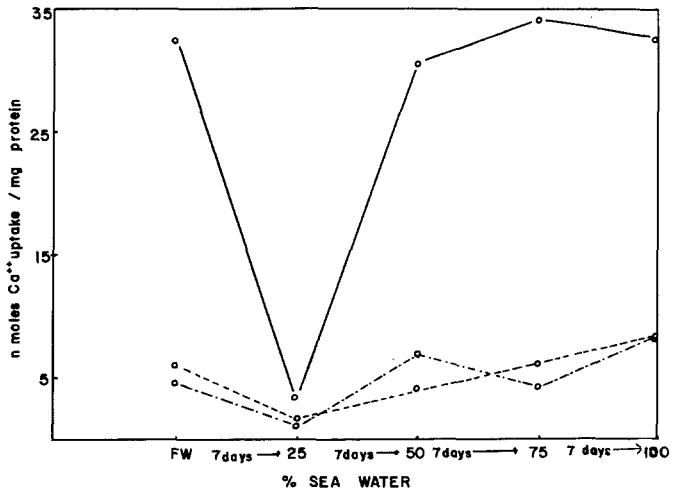

Figure 5. Pattern of energy-dependent calcium uptake during stepwise adaptation to salinity stress. (O————O) Complete system as described previously; (O– – – – –O) system with 30 μM DNP; (O—.—O) system without ATP.

Effect of Stepwise Adaptation to Salinity

As part of screening the effects of salinity stress on mitochondria, the fishes were adapted to increasing salinities in a stepwise manner as described in Materials and Methods. Further, in these experiments, after transfer to a higher salinity, the fish were allowed to acclimate for a period of eight days before their tissues were dissected out and analyzed. This procedure was followed based on a number of reports [4, 5] that a chronic regulative phase which leads to a new steady state is reached around 6–7 days after the stress is introduced.

Respiratory Capacity. There were no significant differences in the mitochondrial content (mg protein/g tissue) oxidation rate, R.C. index, or ADP/O ratio from those obtained for fresh-water fish. The one noticeable difference was that the muscle tissues of salinity-exposed animals were softer in texture.

Energy-dependent Ca^{2+} Uptake. The data are given in Fig. 5. On transfer to 25% sea water, there is a six- to seven-fold decrease in the Ca^{2+} uptake capacity, but this drop is restored to normal levels on subsequent transfers to higher salinities.

Interestingly, the low uptake at 25% sea water was not energy dependent (Table I), suggesting an uncoupled state. Energy coupling is restored subsequently. As is to be expected, the kinetics of uptake (Fig. 2) was very low.

The temperature dependency of this uptake (Fig. 3b) showed a monophasic Arrhenius plot with an activation energy of 20.8 Kcal/mole, about 1.8 times more than the fresh-water fish muscle mitochondria. The pattern was reversed to that of the fresh-water fish during the days 15, 22 and 29.

Energy-Independent Ca^{2+} Uptake. As is seen from Fig. 4, 8-day exposure to 25% sea water changed the binding sites, and also the affinities. A biphasic curve disappears and the monophasic line simulating the fresh-water samples were obtained.

Fatty Acid Composition. A concurrent analysis of fatty acid composition at these stages (T. Vijayalakshmi, unpublished observations) showed that where the fresh-water fish mitochondria had six fatty acid species, after 8 days in 25% sea water, they had only four species. Further exposure to higher salinities reversed the trend.

Time Course of Adaptation to Salinity

The above series of experiments were carried out with the notion that by 8 days under stress, the organism has reached an adaptive phase or a new steady state. To verify this the fishes were transferred to 25% sea water, and samples taken out every alternate day, and calcium uptake by mitochondria studied. Figure 6 shows the data of such an experiment. The Ca^{2+} uptake capacity indeed showed a decrease and reached a minimum on the eighth day, substantiating the results of the earlier experiments. But interestingly, continued exposure resulted in a regain of the activity and by

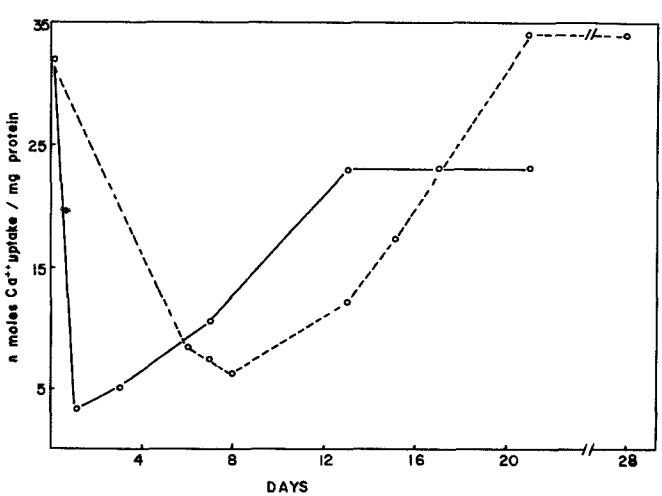

Figure 6. Pattern of energy-dependent calcium uptake as a function of time of exposure to salinity stress (O—O) 25% sea water (8‰ salinity); (O———O) 50% sea water (16‰ salinity).

21 days the full activity was restored. This restoration is also shown in the kinetics of uptake (Fig. 2). Further, the energy coupling is also regained.

The results of these experiments would indicate that what was observed on the eighth day, or up to the eighth day, is indeed the response of the fish to the stress, and the adaptive regulation takes place only later.

Therefore, it was of interest to see whether this response time would depend on the magnitude of the stress. The fish were therefore transferred to 50% sea water from fresh water and samples analyzed every alternate day. Interestingly (Fig. 6), the response to the stress was evident even on the very first day. The low Ca^{2+} uptake of the mitochondria isolated on the first day of exposure was energy-uncoupled. After the first day, the recovery is evident, but at a lower rate till about the 12th day.

Discussion

That the muscle tissue responds to different forms of environmental stress–like low O_2 tension, temperature, and endurance exercises — has been well documented. The general participation of this tissue in regulation of mineral and water metabolism has also been implied by several studies [6]. Specific changes in the Na^+ and K^+ content of the muscle tissue of salmon are seen during its migration from sea water to fresh water. Recently, this response has been traced to the intracellular level, that is, to the mitochondrial level in the case of endurance exercise [7], and also cold acclimation [6]. In this paper, we present evidence to show that muscle mitochondria responds to the salinity stress to which the fish is exposed.

By study of several parameters, it is suggested that the stress has affected the structure or conformation of mitochondria (Scatchard plots, Arrhenius plots, fatty acid composition) and thus also the Ca^{2+} uptake capacity. We have so far no evidence to indicate that the respiratory capacity or oxidative phosphorylation is affected. Other work in this laboratory (Indu Bashyam, unpublished observations) indicates that the swelling–contraction behavior of mitochondria is also affected as a response to osmotic stress. It is therefore interesting to hypothesize on the regulation of the channelization of the energy produced by mitochondria for various functions. A comparative survey carried out by Dr. G. V. Honnappa (to be published) on the energy channelization pattern of different organisms shows that ATP production remains unaltered (utilizing 42% of the total energy produced), whereas the extent of energy transduced for other mitochondrial functions altered from species to species depending upon the physiological variations. We have so far not used any condition where ATP production could be affected (e.g., cold acclimation).

Even after building up a prima facie case for a role of mitochondria in adaptation to salinity, one can only speculate on this obviously complex task of correlating mitochondrial response to the physiological events.

When an aquatic organism is subjected to salinity stress, it enters a nonsteady state, which is exhibited as a change in functional response [4, 8]. Four compensatory devices, namely, escape, reduction, regulation, and adaptation, are available to counteract the detrimental effects of the stress [9, 10]. We believe that in the fish, salinity stress introduces a nonsteady state which lasts until the eighth day with $8^0/_{00}$ salinity (25% sea water) and is evident even on the first day with $16^0/_{00}$ salinity (50% sea water). This nonsteady state is reflected on the mitochondrial function of Ca^{2+} uptake. At this state, the active uptake is very low and most interestingly even the low uptake is energy-uncoupled. Preliminary analyses of endogenous Ca^{2+} levels of these mitochondria (Indu Bashyam, unpublished) showed high values compared to fresh-water fish. Based on these observations the following working hypothesis is derived. As soon as the fish is exposed to high salinity, the intracellular Ca^{2+} level goes up, and in order to regulate this, at least temporarily during the nonsteady state, the mitochondria gets loaded up with Ca^{2+}. This loaded mitochondria is unable to exhibit active uptake in vivo. Subsequent to this period, a regulative phase sets in, and as the ionic stress is relieved due to the compensatory devices which begin to operate, the normal function is restored.

The above hypothesis has not taken into account the involvement of other parameters. The presence of other ions in sea water like Na^+, K^+, and Cl^-, which have been shown to influence Ca^{2+} uptake in in vitro studies [11–15], would also be expected to exert their influence in vivo. We have also shown that mitochondria preloaded with Ca^{2+} release these ions with increasing concentrations of Na^+ in the incubation time. Experiments are now in progress to ascertain whether the results obtained with sea water could be simulated by rearing the fish in single salt solutions.

In conclusion, one can state that the studies reported here have thrown open a new avenue of research in the area of mitochondrial energy transductions and dynamics. It is hoped that our studies on the mitochondria from gill and kidney, now in progress, will provide substantiating data on the role of mitochondria in biochemical adaptation.

References

1. G. V. Honnappa, R. H. Sulochana, and J. Jayaraman, *J. Bioenergetics,* **7** (1975) 149.
2. O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. S. Randall, *J. Biol. Chem.,* **193** (1951) 265.

3. B. Reynfarje and A. L. Lehninger, *J. Biol. Chem.,* **244** (1969) 584.
4. F. P. Conte, *Fish Physiology,* Vol. I, W. S. Hoar, and D. J. Randall, (eds.), Academic Press, London, (1969) p. 241.
5. W. J. Holmes and E. N. Donaldson, in *Fish Physiology,* Vol. I, W. S. Hoar and D. J. Randall (eds.), Academic Press, London, (1969) p. 1.
6. H. Hensel and G. Hildebrandt, in *Handbook of Physiology,* Section 4, C. G. Wilber (ed.), American Physiological Society, Washington D.C., (1964) p. 73.
7. J. O. Holloszy, *Medicine and Science in Sports,* **7(3),** (1975) 155.
8. C. R. Hickman and B. F. Trump, in *Fish Physiology,* Vol. I, W. L. Hoar and D. J. Randall (eds.), Academic Press, New York, (1969) p. 91.
9. O. Kinne, *Nether. J. Sea. Res.,* **3** (1966) 222.
10. C. L. Prosser, in *Molecular mechanisms of temperature adaptation,* C. L. Prosser (ed.), American Association for the Advancement of Science, Washington D.C., (1967) p. 351.
11. C. S. Rossi, E. Carafoli, Z. Drahota, and A. L. Lehninger, in *Regulation of Metabolic Processes in Mitochondria,* J. M. Tager, S. Papa, E. Quagliariello, E. C. Slater (eds.), Elsevier Publishing Co., Amsterdam (1966) p. 329.
12. A. L. Lehninger, *Ann. N.Y. Acad. Sci.,* **137** (1966) 700.
13. H. Dransfeld, K. Great, A. Schorn, and B. T. Ting, *Biochem. Pharmacol,* **18** (1969) 1335.
14. E. Carafoli, *Biochemie Extrait du Tome,* **55** (1973) 755.
15. E. Carafoli, *Biochem. Soc. Symposium,* **39** (1974) 89.