Metabolic biochemistry of water- vs. air-breathing osteoglossids: heart enzymes and ultrastructure

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The ultrastructure of the inner myocardium of aruana, an osteoglossid water breather, and *Arapaima*, an air-breathing Amazon relative, was compared. The aruana heart was laden with glycogen granules while *Arapaima* heart was fat loaded. Associated with air breathing in *Arapaima*, the ultrastructure of the inner myocardium displayed abundant mitochondria, clearly differentiated into myofibrillar and peripheral populations. As many of the mitochondrial characteristics of *Arapaima* resembled those in the mammalian heart, it was postulated that the inner myofibrillar mitochondria are probably specialized for oxidative metabolism as is the case also in the mammalian heart, while subsarcolemmal mitochondria were specialized for the exchange of materials with the blood. In contrast with the heart of the air breather, in aruana the inner myocardium contained two cell types. Type I cells like those in *Arapaima* myocardium were specialized for aerobic metabolism, displaying abundant mitochondria mostly myofibrillar in location and ample glycogen granules as a potential substrate source. Type II cells by comparison contained fewer mitochondria, but were rich in glycogen granules, and appeared specialized for anaerobic metabolism.

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L'examen du myocarde interne chez l'aruana, ostéoglossidé à respiration aquatique, et chez *Arapaima*, poisson parent à respiration aérienne de l'Amazone également, a permis d'établir certaines comparaisons. Le cœur de l'aruana est tapissé de granules de glycogène, alors que celui d'*Arapaima* est rempli de graisses. Chez *Arapaima*, l'ultrastructure du myocarde est associée à la respiration aérienne: il y a de nombreuses mitochondries bien différenciés en deux populations situées l'une dans la région des myofibrilles, l'autre en position périphérique. Comme les mitochondries se rapprochent par plusieurs de leurs caractéristiques de celles d'un cœur de mammifère, il est permis de croire que les mitochondries de la région des myofibrilles sont probablement responsables, comme c'est le cas dans le cœur des mammifères, du métabolisme d'oxydation et que les mitochondries situées sous le sarcolemme sont sans doute spécialisées dans les échanges avec le sang. Chez l'aruana, le myocarde interne comporte deux types de cellules. Les cellules de type I, comme les cellules du myocarde d'*Arapaima*, servent au métabolisme aérobique et comportent donc beaucoup de mitochondries, surtout dans la région des myofibrilles, et de gros granules de glycogène, substrats potentiels. En revanche, les cellules de type II contiennent moins de mitochondries, mais sont riches en granules de glycogène; elles sont probablement adaptées au métabolisme anaérobique.

[Traduit par le journal]

Introduction

At an organ level, there are in theory at least three ways of dealing with problems of periodic hypoxia. Firstly, the anaerobic capacity of the organ can be adjusted by changes in the amounts of storage substrate (glycogen) with no change in cell type or in enzyme levels. Secondly, the potential can be adjusted by changes in the amounts and kinds of key glycolytic enzymes, again with no change in cell type. Thirdly, the organ can be differentiated to include some cell types specialized for anaerobic metabolism (by adjustments in substrate storage or enzyme amounts or both) and some specialized for aerobic metabolism. In vertebrate skeletal muscle, permutations and combinations of all three adaptive mechanisms are probably utilized by some species somewhere (see Holloszy and Booth 1976), the differentiation into red (aerobic) and white (anaerobic) muscles in many teleosts being particularly clear and pronounced (see Driedzic and Hochachka 1976; Guppy *et al.* 1977; Johnston *et al.* 1975). In the heart, the adaptive 'options' appear to have been reduced. Hearts of diving marine mammals and turtles, for ex-

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ample, show an increased capacity for anaerobic glycogenolysis which is expressed by storage of high amounts of glycogen, by maintenance of high activities of glycolytic enzymes, and by modifications in key kinetic properties of glycolytic enzymes (see Hochachka and Storey (1975) for a recent review). These characteristics, presumably of advantage during prolonged diving when O_2 supplies are at a premium, are achieved with no known differentiation and development of specialized cell types. For this reason, in our comparison of heart metabolism in water- vs. air-breathing osteoglossids, our finding that two myocardial cell types (one highly aerobic, the other relatively anaerobic) were associated with retention of water breathing was rather surprising.

As in our studies of skeletal muscles (Hochachka *et al.* 1978), we used enzymatic and ultrastructural methods to study the inner myocardium of aruana, an osteoglossid water breather, and *Arapaima*, a related Amazon air breather. We found three strategies for dealing with hypoxia stress in aruana myocardium. In the first place, aruana heart stored high amounts of glycogen; secondly, the levels of key glycolytic enzymes (phosphofructokinase (PFK) and pyruvate kinase (PK)) were high; and thirdly, aruana inner myocardium displayed two cell types. Type I cells displayed more mitochondria than type II cells. Both cell types contained abundant glycogen but no intracellular triglyceride. In the air breather, *Arapaima*, the inner myocardium consisted of only one cell type, rich in mitochondria and fat, but also containing ample glycogen. The ultrastructure and enzymology of *Arapaima* heart indicate a metabolic organization that, like in diving marine mammals and turtles (Hochachka and Storey 1975), can oscillate between glycogen fermentation when O_2 is limiting and fat catabolism when O_2 supplies are abundant.

Methods and Materials

Experimental Animals

Adult representatives of the obligate water breather, *Osteoglossum bicirrhosum* (termed aruana in this paper), and its air-breathing relative, *Arapaima gigas* (termed simply *Arapaima* in this paper) were captured and held on board as described by Hochachka and Randall (1978).

Tissue Preparation

For enzyme studies, active and apparently healthy animals were netted and killed by decerebration. The heart was quickly excised, weighed, then homogenized in 50 m*M* imidazole buffer, pH 7.0. The homogenate, usually 1:10 w/v basis, was well stirred, then spun at 15 000 × *g* to remove cellular debris. The resulting supernatant solution was used directly as an enzyme source for determining maximum enzyme activities. Conditions of assay are given by Hochachka *et al.* (1978). Activity was monitored at 340 nm in a Unicam SP 1800 recording spectrophotometer at 25°C, pH 7.0, in 50 m*M* imidazole buffer, with the exception of citrate synthase, which was assayed at 410 nm in 50 m*M* Tris–HCl buffer, pH 8.0.

Tissue Preparation for Electron Microscopy

For microscopy, small samples of cardiac muscle were quickly excised from two freshly decerebrated fish and placed in glutaraldehyde; the cardiac muscle pieces were then cut with a sharp razor into smaller cubes (about 1–2 mm^3) and subsequently handled as described by Hulbert *et al.* (1978).

Reagents and Coupling Enzymes

All organic metabolites and purified coupling enzymes were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents used were in all cases of analytical grade.

Results and Discussion

General Observations

The osteoglossid heart, like that in other primitive vertebrates, is composed of four chambers, with blood flowing anteriorly through the sinus venosus, the atrium, the ventricle, and the conus arteriosus. The ventricle is the thick-walled major contractile portion of the heart, directing blood through the conus and into the ventral aorta. In both aruana and *Arapaima* the ventricle is covered by a continuous sheet of simple squamous epithelium forming the external epicardium. Underlying the epicardial sheet is the myocardium which in the osteoglossids as in many other teleosts consists of outer and inner layers. The outer layer, supplied with a coronary circulation, in aruana consists of small-diameter (6–8 μm) cells rich in mitochondria and glycogen, both largely myofibrillar rather than peripheral in location. The myofibrils in the inner myocardial layer in both species are organized into trabeculae, the so-called "spongy" layer of the teleost heart (see Kilarski 1964*a*, 1964*b*; Lemanski *et al.* 1975). The spongy layer in both osteoglossids is separated from the blood in the inner ventricular chamber by an epithelial layer termed the endocardium.

In a 1-kg aruana, the heart constitutes on average only about 0.025% of body weight; in *Arapaima* of similar body size, the ventricle alone is about 0.1% of body weight, compared with about 0.5% in man. The relatively large heart of *Arapaima* is tightly enclosed in connective tissue that is loaded with lipid (Fig. 1). This lipid-laden connective tissue, containing lipase activity (J. Patton, personal communication), probably serves as an important supplementary source of fuel (as free fatty acids?) for a relatively vigorous fat catabolism in the heart of the air breather (discussed further below). In aruana, this feature is not evident, nor as we shall discuss below, is there any intracellular triglyceride present. These gross observations encouraged us to look for more basic differences in the ultrastruc-

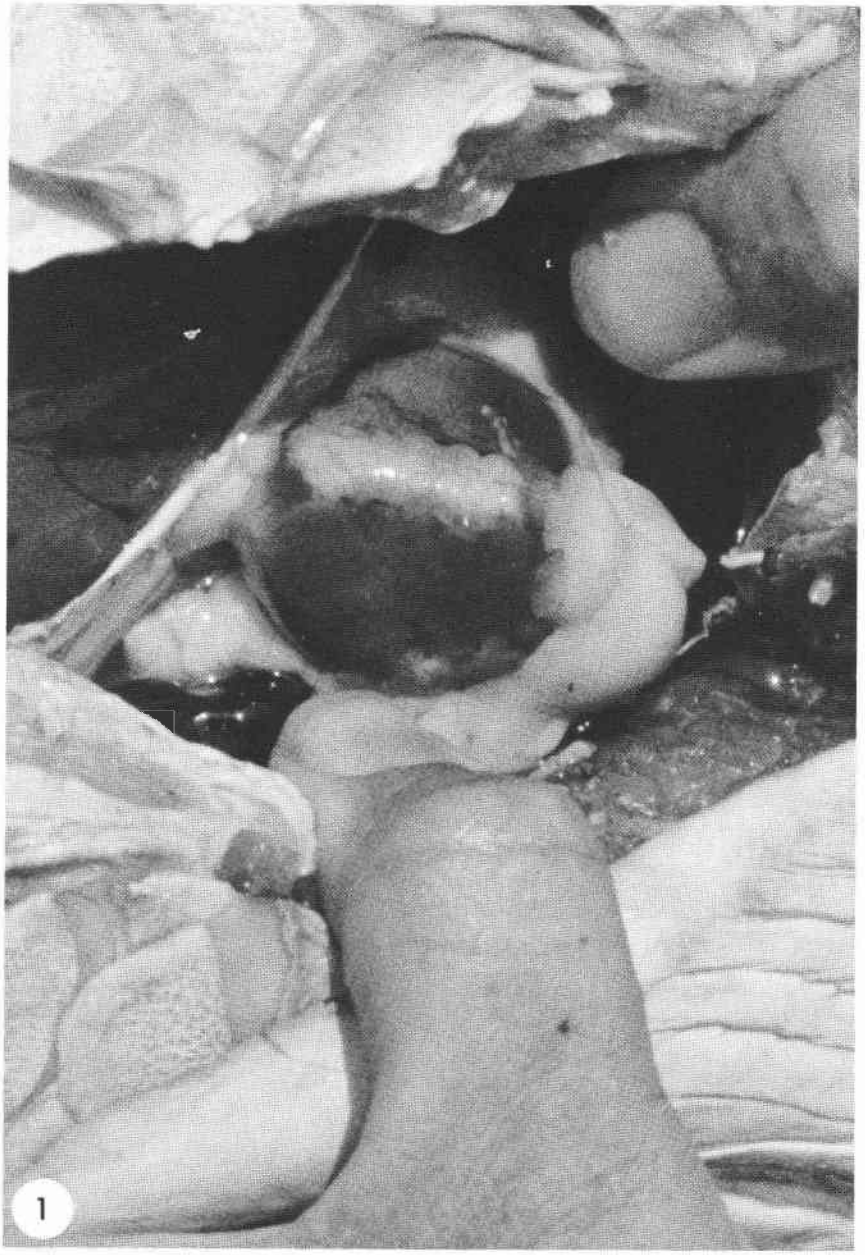
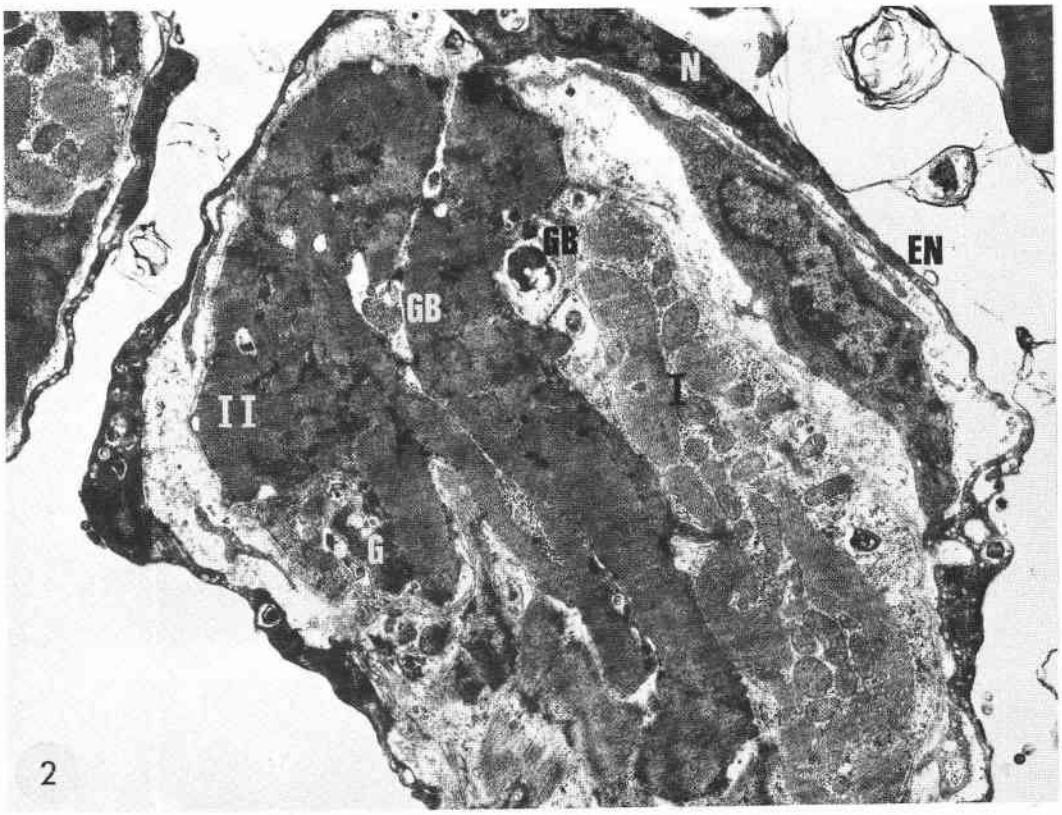


FIG. 1. An overview of the *Arapaima* heart showing the well-developed ventricle and the surrounding adipose-loaded connective tissue, containing triglyceride lipase activity (Patton, personal communication) and a vascular supply.

ture of the two hearts. We chose to compare and contrast the ultrastructure of the inner myocardium in the ventricles of the two species.

Aruana Inner Myocardium

Singly the most dramatic observation made in our studies of the aruana inner myocardium is the occurrence of two cell types (Fig. 2). Type I cells usually appear to be less electron absorbing and hence appear lighter on electron micrographs than type II cells. Type I cells are usually bundled or packaged in trabeculae along with the type II cells, the trabeculae being surrounded by a nucleated thin epithelial sheet, the endocardium (Fig. 2). Based on multiple electron microscope scans, the ratio of type I to type II cells in different trabeculae varies, being about 1:2 on average. Although some trabeculae are filled with only type II cells (Fig. 3),



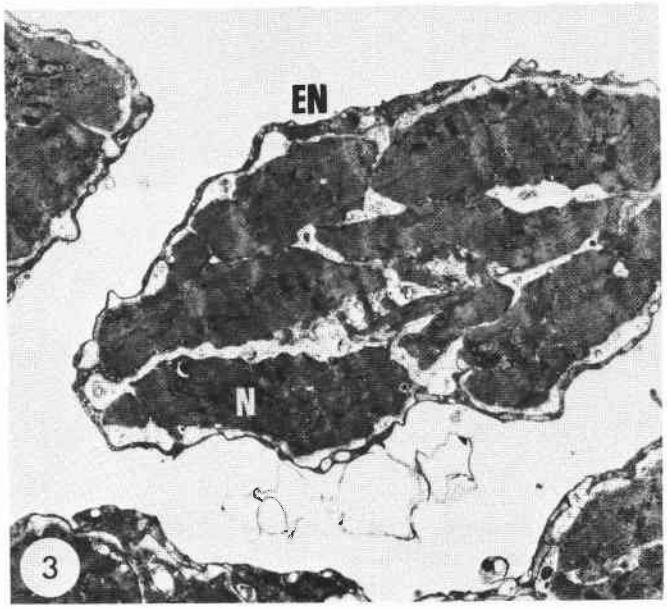


FIG. 2. Aruana inner myocardium. Electron micrograph (× 8625) showing a trabecular cross section. A thin epithelial, nucleated layer, the endocardium (EN) separates the cardiac muscle cells from the inner chamber. Aruana trabeculae are composed of two distinct types of cells. Type I cells are less electron dense than type II cells. They contain greater numbers of mitochondria, predominantly myofibrillar in position. Both cell types display numerous monoparticulate glycogen granules, but type I cells typically have less glycogen than type II cells. Periodically, glycogen bodies are seen (see Hochachka and Hulbert (1978) for a further discussion of glycogen bodies). FIG. 3. Aruana inner myocardium. Electron micrograph (× 5000) of a trabecula formed entirely from type II cells. Trabeculae formed from only type I cells do not occur, or at least were never observed in multiple blocks examined. EN, endocardium; N, nucleus.

we found no trabeculae formed from only type I cells. About 3–5 cells occur per trabecula, compared with about 25 in the case of *Pleuronectes* (Santer and Cobb 1972), a difference between the two species that may be related to the larger size of the aruana cells (diameter of 7.5–12.5 μm compared with about 4.5 μm in *Pleuronectes*). In the Japanese madaka, the myocardial cells are about 6–9 μm in diameter (Lemanski *et al.* 1975) and the number of cells per trabecula are intermediate between aruana and *Pleuronectes*.

The type I cells differ from type II in a number of important ways. Thus, although mitochondria occur in both types, they are more abundant in type I cells. The mitochondria assume a variety of shapes, oval being most common (see Figs. 2, 4, 6) and are filled with rather thickened, almost finger-like cristae that are of medium density. The mitochondria are usually myofibrillar in position (Fig. 4). Infrequently they can also be observed peripherally. These observations suggest that type I cells are specialized for oxidative metabolism, hence it is instructive that they contain no intracellular lipid. They do, however, contain ample glycogen, which is stored as monoparticulate granules and often packaged tightly amongst or against mitochondria.

Although type I cells contain plenty of glycogen, type II cells appear to contain even more. In fact, in type II cells the abundance of monoparticulate glycogen granules undoubtedly contributes to the greater electron absorption by these cells. As in type I cells, most glycogen granules are packaged between myofibrils (Fig. 6). These observations suggest that type II cells, though capable of oxidative metabolism, are primarily specialized for anaerobic metabolism with glycogen serving as the chief carbon and energy source for both processes.

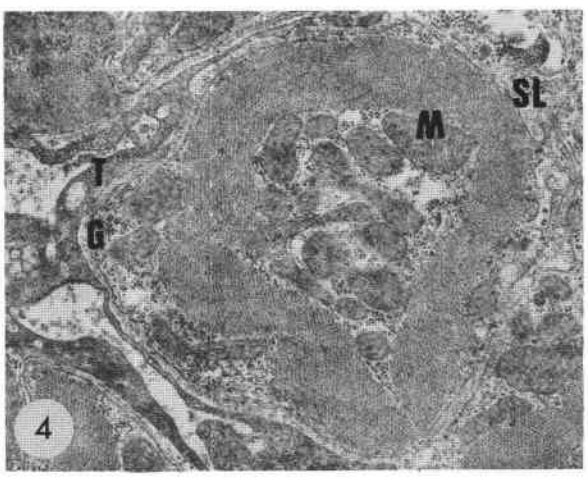
Periodically, peculiar electron-transparent regions or 'holes' occur surrounded by a sea of glycogen granules (Fig. 2). In some cases these 'holes' contain electron-absorbing material while in other cases they do not. Similar structures in lungfish and *Synbranchus* hearts are clearly identifiable as depleted glycogen bodies (Hochachka and Hulbert 1978).

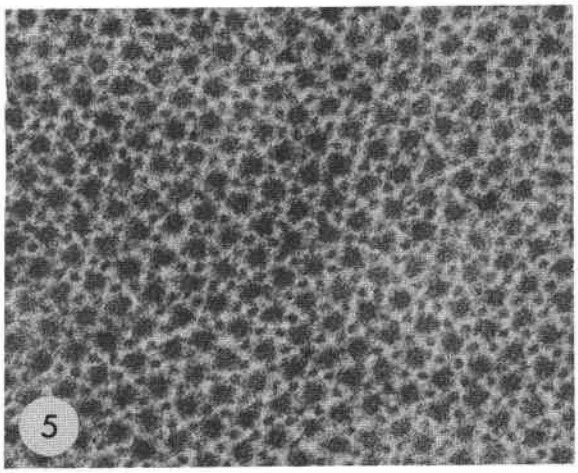
One of the primary differences first noted between type I and type II cells in the aruana myocardium was the overall electron opacity of electron micrograph sections. We reasoned that one factor contributing to this characteristic could be the ratio of actin:myosin. However, from multiple grid and micrograph scans it appears that in all type I cells and most type II cells, the ratio of actin:myosin is on average 6 (Fig. 5), as is commonly found in vertebrate muscle (see Smith 1972). Hence, other factors must account for the different electron-absorbing properties of the two cell types.

In other regards, type I and II cells appear to be rather similar. Intercalated discs are evident in electron micrographs of both cell types. These take the form of desmosomes (not shown), and interfibrillar junctions (entirely similar to those shown for *Arapaima* in Figs. 8 and 9), but no examples of the nexus interaction could be found, in agreement with Lemanski *et al.* (1975). Although the sarcoplasmic reticulum (SR) is present, in neither cell type is it extensive. As observed in other teleost hearts (see Lemanski *et al.* 1975), transverse tubule systems are not evident. It is widely accepted that T-systems in muscles of higher vertebrates provide the contractile elements with communication channels to the external cellular environment. Lemanski *et al.* (1975) suggest that in the teleost heart subsarcolemmal cisternae (thought to be continuous with the SR), coupled with the peripheral localization of the myofibrils, preclude the need for conventional T-systems. Such a conclusion does not contradict current concepts of excitation–contraction coupling and associated calcium flows (see Fabiato and Fabiato (1977) for a review), but it does suggest that there may be a somewhat different way of getting the impulse into the muscle cell initially.

Arapaima Inner Myocardium

Although by comparison with aruana, the *Arapaima* inner myocardium is composed of cells of similar size (about 8–12 μm in diameter), it differs markedly in containing only one cell type (Fig. 7). The trabeculae form a spongy layer as in aruana but on average a larger number (five or more) cells contribute to trabecular formation. The trabeculae are separated from the inner blood chamber by a thin sheet of epithelium, the endocardium, which is nucleated and vacuolated, but does not show the excessive pinocytotic activity noted in other teleosts (Lemanski *et al.* 1975). SR is present, but as in aruana and in other teleosts, it is not well developed; T-systems are again absent. Intercalated discs (Figs. 8 and 9) are readily evident and appear more numerous than in aruana. They may occur as desmosomes or as interfibrillar junctions, but as in aruana, no clear examples of the nexus type of cell–cell interaction could be found. In appropriate planes of section, these intercalated discs appear to be continuous with Z-bands of myofibrils from adjacent cells. In addition to the above relatively normal cell–cell contacts, an interdigitating membrane interaction periodically occurs (Fig. 8, inset) a feature not seen in mammalian myocardium. (See Hochachka and Hulbert (1978) for a discussion of a





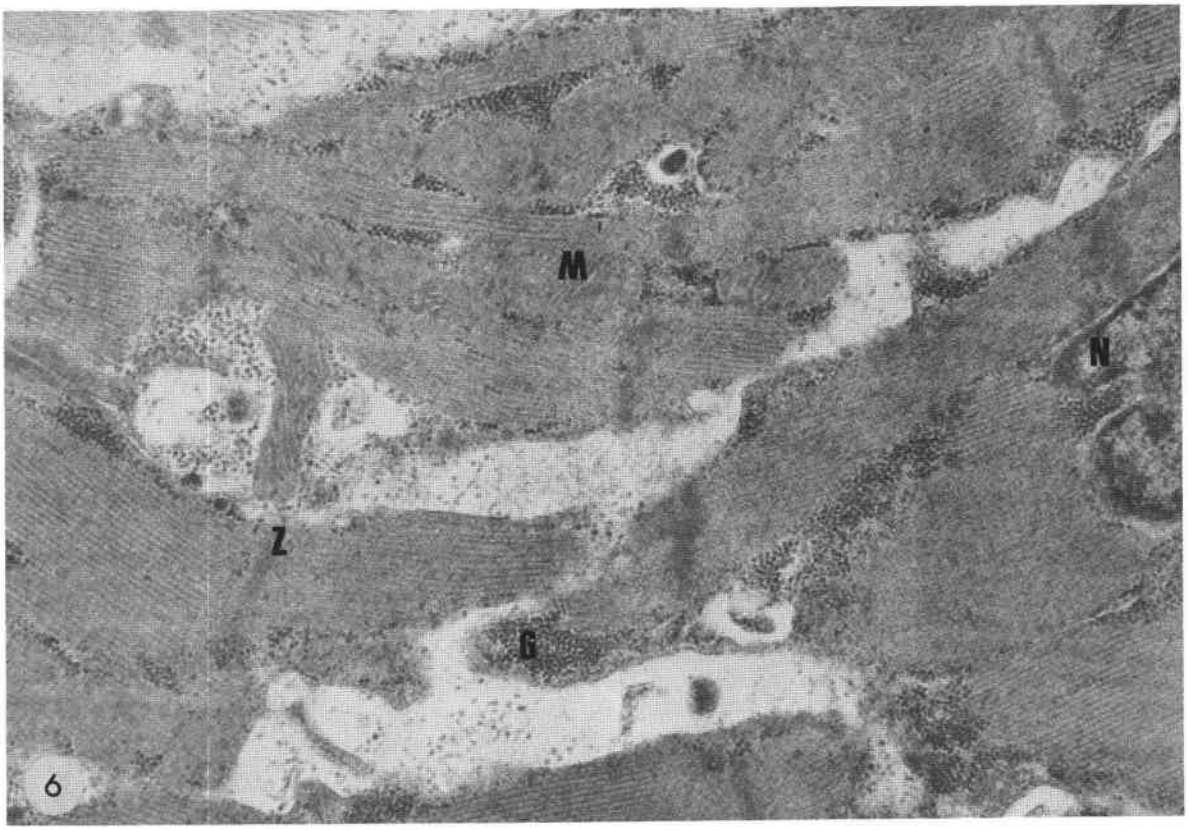


FIG. 4. Electron micrograph (× 12 500) of aruana inner myocardium showing a cross section of cardiac muscle cell. The mitochondria (M) are numerous, are predominantly myofibrillar in localization, and usually assume a variety of shapes. G, glycogen; T, trabecula; SL, sarcolemma. FIG. 5. Electron micrograph (× 135 000) of aruana inner myocardium showing a cross section of a type I cell. The typical actin:myosin ratio of 6:1 can be readily observed. FIG. 6. A tangential section (× 23 900) of aruana type II cell demonstrating abundant glycogen and high electron density of these cells. The high electron density greatly reduces contrast in these electron micrographs. Note that the density of Z-lines is only slightly greater than that of the myofibrils, in contrast with *Arapaima* (see Fig. 7). G, glycogen; M, mitochondria; Z, Z-line; N, nucleus.

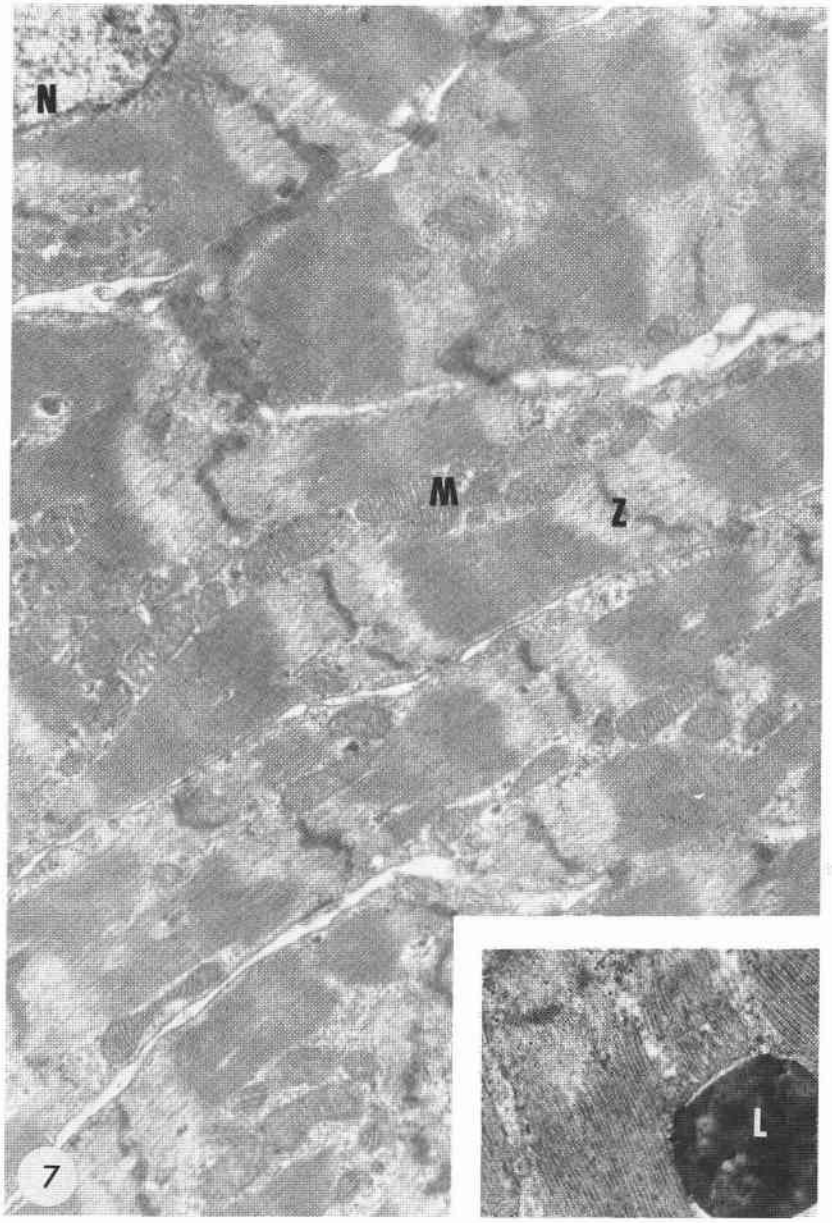
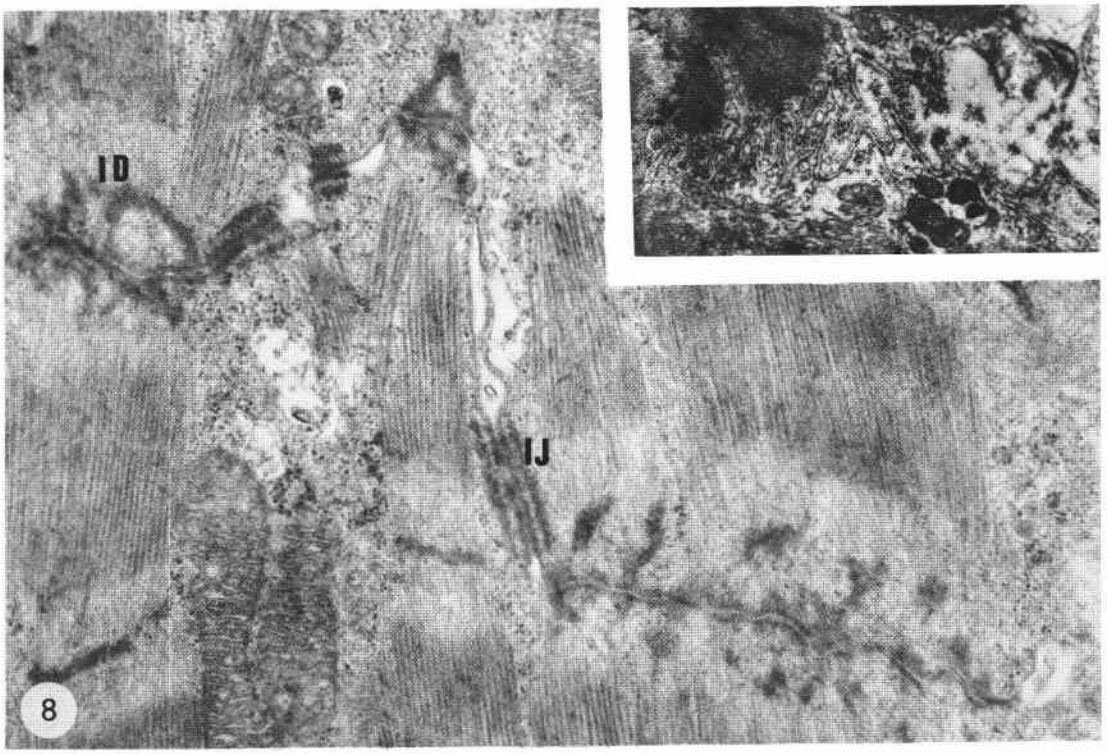


FIG. 7. Electron micrograph (× 15 600) of *Arapaima* inner myocardium showing a slightly tangential section through a trabecula. Trabeculae in *Arapaima*, unlike aruana, are composed of only one muscle cell type. Mitochondria (M) are fairly abundant, occurring either in myofibrillar or peripheral positions. The entire trabecula is surrounded by a thin, nucleated epithelium termed the endocardium. Glycogen granules (G) are evident but are not abundant. Lipid droplets (L) are also frequently noted (Fig. 7, inset; × 39 200). Z, Z-line.

similar structure in lungfish and *Synbranchus* hearts.)

As in type I cells of aruana myocardium, *Arapaima* myocardial cells are rich in mitochondria, but unlike the situation in aruana, these seem differentiated in *Arapaima* for a wider variety of functions. Thus, in terms of position in the cell, the mitochondria firstly can be myofibrillar, essentially fully enclosed by myofibrils (Fig. 7); in the mammalian heart, myofibrillar mitochondria seem most active in oxidative metabolism (Wilson *et al.* 1977), and it is reasonable to guess that these have been similarly specialized in *Arapaima*. In addition, however, in *Arapaima* inner myocardium, mito-



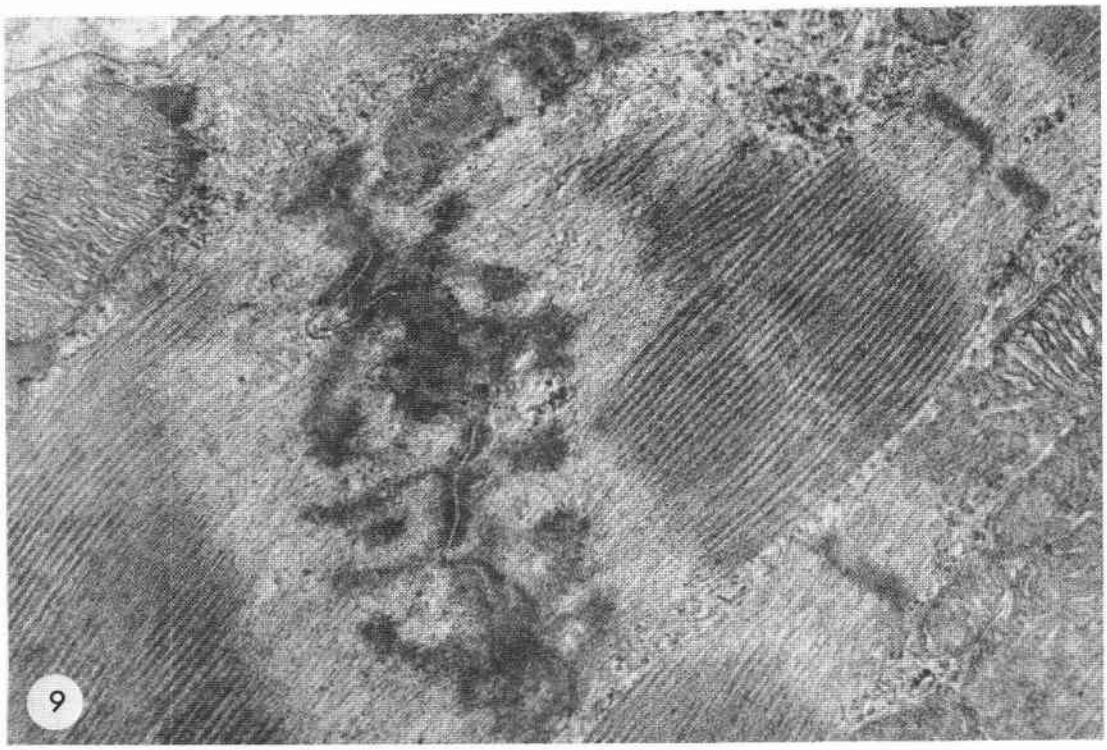


FIG. 8. Longitudinal section (× 18 600) of *Arapaima* heart muscle showing the structure of intercalated disc regions and interfibrillar junctions. ID, intercalated disc; IJ, interfibrillar junction. FIG. 8, inset. Electron micrograph of *Arapaima* inner myocardium (× 9 800) showing interdigitating of adjacent sarcolemmal membranes, but not forming a true junctional complex. FIG. 9. Higher magnification of *Arapaima* heart muscle intercalated disc (× 42 000).

chondria can also be found peripherally, adjacent to the sarcolemma (Figs. 7, 9), or packaged into peripheral papillae (not shown) that project from trabeculae into the inner chamber. In vivo these peripheral mitochondria would presumably be separated from the blood only by a thin endocardium. As in the mammalian heart (Frank and Langer 1974; Wilson *et al.* 1977), these peripheral mitochondria seem particularly well positioned for the exchange of material (O_2, CO_2, Ca^{2+}, metabolites) between the mitochondria, the cytosol, and the blood.

In the degree to which these mitochondria seem differentiated for specialized functions, the *Arapaima* inner myocardium more closely resembles the mammalian heart than the typical teleost heart or the aruana myocardium. This rather marked similarity between *Arapaima* and mammalian myocardium is further underlined by the presence of abundant intracellular triglyceride in *Arapaima*. The lipid droplets most often appear to be located near mitochondria (Fig. 7, inset), but they are also sometimes seen between myofibrils and near nuclei. Although Kilarski (1964*a*) observed small amounts of intracellular lipid in the lamprey myocardium, it is not usually observed in any abundance in teleost myocardium (see Kilarski 1964*b*; Lemanski *et al.* 1975). Nor is it found in aruana heart. In contrast, glycogen granules are common in teleost hearts, though not usually as abundant as we observed in aruana type II cells (see Fig. 2). In *Arapaima*, although the inner myocardium does contain glycogen granules, they are by no means as abundant as in aruana (compare for example Figs. 2 and 7). From these ultrastructural studies it is difficult to avoid the conclusion that in *Arapaima* heart, glycogen, and fat are the main fuel sources for cardiac work; in aruana, by contrast, glycogen is by far the more important substrate source, intracellular fat in fact being excluded. Measurements of activities of selected enzymes in energy metabolism are consistent with this view.

Enzyme Profiles

Interestingly, in view of the ultrastructural differences in the hearts of *Arapaima* and aruana, the enzyme profiles for the two organs (Table 1) are remarkably similar. The primary differences appear in the levels of PFK, PK, and lactate dehydrogenase (LDH), which occur at about 2-, 3-, and 1.2-fold higher levels in aruana heart homogenates than in *Arapaima* heart. If these enzymes are taken as a rough indication of the potential for anaerobic glycolysis, then it is clear that this capacity in aruana heart may be substantially greater than in *Arapaima*. The difference in hypoxia tolerance of the two hearts may in fact be greater than implied by the enzyme measurements, since in aruana the activities extractable from two fiber types are assayed together. It is probable that the activities of the glycolytic enzymes in type II fibers of aruana are in fact higher than indicated in Table 1 for crude homogenates.

TABLE 1. Heart enzyme levels in aruana and *Arapaima*, in micromoles per minute per gram wet weight (number of determinations in parentheses; range below each average)

Enzyme and role	Aruana	*Arapaima*
Anaerobic metabolism		
PK	144(2)	52(3)
	135–152	49–57
LDH (low pyruvate)	409(3)	367(3)
	332–509	300–405
LDH (high pyruvate)	229(3)	245(3)
	205–243	194–325
Aerobic metabolism		
MDH	248(4)	389(3)
	191–318	223–486
CS	11.0(2)	10.3(3)
	10.7–11.3	5.5–15.7
GDH	2.86(3)	3.44(3)
	1.9–3.4	2.8–4.5
GOT	71.4(2)	48.1(3)
	57.9–84.9	33.1–62.4
Other functions		
G6PDH*	0.2(1)	1.2(3)
		0.75–2.0
PFK*	10.7(4)	5.7(3)
	6.4–18.6	0.7–8.9
FDPase*	0.25(2)	0.54(3)
	0.14–0.35	0.41–0.81
Aldolase	11.1(2)	10.9(3)
	2.8–19.5	7.3–16.2
αGPDH*	0.41(3)	0.46(2)
	0.1–1.0	0.41–0.51
AMP deaminase	0.18(2)	0.48(2)
	0.42–0.34	0.39–0.57

*ABBREVIATIONS: G6PDH, glucose-6-phosphate dehydrogenase; PFK, phosphofructokinase; FDPase, fructose diphosphatase; αGPDH, alpha-glycerophosphate dehydrogenase.

The occurrence of similar activities of enzymes in aerobic metabolism, such as citrate synthase (CS) and glutamate dehydrogenase (GDH), would be expected from a similar mitochondrial abundance in the two hearts. However, the malate dehydrogenase (MDH) levels in the two hearts deserve a brief explanatory comment. The high and similar MDH levels may be due to a similar abundance of mitochondria. On the other hand, cytosolic MDH may take on a role in redox regulation during hypoxic stress; if type II fibers contributed in a major way to the high activity in aruana this may also explain the presence of unusually high aspartate aminotransferase (GOT) levels, since in hypoxic periods these two enzymes may function in aspartate fermentation to malate (then succinate)

with the consequent oxidation of cytoplasmic NADH (see Collicutt and Hochachka (1977) for a recent discussion of this role in invertebrate heart; see Hochachka and Storey (1975) for its potential role in vertebrate hypoxia-adapted hearts). Consistent with this view is the observation that, although GOT is abundant in both hearts, in aruana a larger fraction of the total activity is cytosolic (Hochachka and Guderley, unpublished data). In *Arapaima* heart, on the other hand, GOT appears to play important roles in hydrogen shuttling via the malate–aspartate cycle and perhaps in the regulation of Krebs cycle intermediates during transition from glycogen to fat metabolism or vice versa.

Finally, a comment should be made on the LDH activities present in these osteoglossid hearts. On kinetic criteria, myocardial LDH activity in both *Arapaima* and aruana consists predominantly of H-type subunits, sensitive to inhibition by high pyruvate levels. The high LDH present in the two hearts could be utilized during anaerobic metabolism (as may periodically be required in hypoxic periods) or for the oxidation of lactate produced elsewhere (in white muscle, for example) and delivered to the heart as a carbon and energy source, a view fully supported by the observation that lactate is an excellent substrate for subcellular preparations of myocardium from both *Arapaima* and aruana (Hochachka and Schneider, unpublished data).

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