ENERGY LEVELS AND ANAEROBIC ENDPRODUCTS IN THE BRAINS OF TWO SPECIES OF TELEOST FISH AT DEATH IN ANOXIC WATER

by

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(ABSTRACT)

The brain of fish, as in other vertebrates, is responsible for many functions basic to life and is also thought to be an anoxia-sensitive tissue. Therefore, during anoxia, the maintenance of energy within the brain is of paramount importance to the survival of the animal. Studies concerning energy levels and storage and the use of anaerobic metabolism in fish brains following exposure to anoxia are lacking.

Rainbow trout (Salmo gairdneri) and brown bullhead catfish (Ictalurus nebulosus) occupy ecologically distinct habitats. Their tolerance of anoxia is different; trout survived 12 minutes while bullhead survived 62 minutes in anoxic water. Brains from control and anoxia-exposed trout and bullheads were analyzed using enzymatic assays and high pressure liquid chromatography (HPLC).

Control bullhead brains had higher concentrations of glycogen, ATP, CrP, and glucose than control trout. With anoxia, bullheads showed a significant decrease in ATP, CrP, and glycogen with no change noted for glucose, ketone bodies (beta-hydroxybutyrate and acetoacetate), or alternative anaerobic endproducts (succinate, alanine,

propionate, isobutyrate, isovalerate, and ethanol). Lactic acid increased two-fold with anoxia. The bullhead was able to generate ATP by depleting its CrP stores and through classical anaerobic glycolysis. Death was most likely due to an inability to maintain ATP levels. Catfish may survive anoxia longer than trout in part due to greater fuel stores.

Rainbow trout brain stored approximately one-sixth the amount of glycogen as bullheads. With anoxia, these stores were depleted but there was no significant decrease in ATP, CrP, or glucose; the alternative endproducts also did not change. There was a 100% increase in lactic acid, suggesting that anaerobic glycolysis helped maintain ATP levels. Death may be due to factors other than ATP depletion such as lactic acid injury and increased intracellular free calcium.

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1. INTRODUCTION

The brain of fish, as in any other vertebrate, is responsible for many functions basic to life, such as integration of information, coordination of movements, maintenance of tone, and control of breathing (Marshall and Hughes 1980; Shelton 1970; Smith 1982). The sensitivity of the mammalian brain to oxygen deprivation is well-known; the brain of fish is also thought to be an anoxia-sensitive tissue (Hochachka 1985; McDougal et al. 1968). Therefore, during anoxia, the maintenance of energy within the brain is of paramount importance to the survival of the animal. However, the majority of research concerning fish and environmental hypoxia or anoxia has dealt with energy levels and anaerobic metabolism in other tissues such as muscle (Burton and Heath 1980; Driedzic and Hochachka 1975; Johnston 1975a,b; Jorgensen and Mustafa 1980a,b; Smith and Heath 1980; Thillart et al. 1982) and blood (Burggren and Cameron 1980; Heath and Prithchard 1965; Soivio et al. 1980; Weber and Lykkeboe 1978), or with the whole body (Johnston and Bernard 1983; Thillart et al. 1976; Wokoma and Johnston 1983). Studies concerning changes in energy levels and storage, and the use of anaerobic metabolism in fish brains following exposure to anoxia are lacking (see Bandurski et al. 1968; McDougal et al. 1968; Rovainen et al. 1969).

During anoxia the brain must produce energy in the form of adenosine triphosphate (ATP) so that basic life functions can be maintained. Unlike muscle tissue, the limited information available suggests that brain tissue in reptiles and fish exhibits a marked reduction in ATP levels with anoxia. Lack of metabolic energy is cited for the failure of the central nervous system in the brains of turtles and goldfish (Belkin 1968; McDougal et al. 1968; Sick et al. 1984).

The brain of mammals depends almost exclusively on the aerobic catabolism of glucose for the production of ATP. Fish brains appear to use glucose to generate energy under normoxic and anoxic conditions (McDougal et al. 1968; Shaffi 1982). In the absence of glucose, the mammalian brain can utilize ketone bodies (acetoacetate and beta-hydroxybutyrate) as an energy source (Lehninger 1982). The role ketone bodies play in the brains of fish is not known. This study provides some baseline data, as well as possible functions, of ketone bodies in the brain of fish during normoxia and anoxia.

Creatine phosphate (CrP) is a storage form of high-energy phosphates which can be used in the generation of ATP. Fish and reptile brains appear to store large amounts of CrP (McDougal et al. 1968; Rovainen et al. 1969; Sick et al. 1984); depletion of these stores is reflected in

decreased levels of ATP (Jorgensen and Mustafa 1980b). Fish brains are reported to store large amounts of glycogen (Breer and Rahmann 1974). Reduction in glycogen content has been reported in fish and reptile brains during anoxia (McDougal et al. 1968).

Lactic acid is the end-product of classical anaerobic glycolysis. Formation of lactic acid results in the generation of two molecules of ATP, a small fraction of the energy available if glucose were completely oxidized to carbon dioxide and water. Although seemingly inefficient, anaerobic glycolysis appears to be the predominant pathway in the brains of fish and reptiles under anoxia according to several studies (Belkin 1968; McDougal et al. 1968; Sick et al. 1984). It has been suggested that ethanol is formed from lactic acid in the muscle of goldfish. The proposed pathway would not generate ATP but rather would maintain the redox balance and decrease the build-up of acid end-products (Johnston and Bernard 1983; Shoubridge and Hochachka 1980). No data concerning the production of ethanol by the brain of fish exist, but this study examines the role of ethanol during anoxia.

Certain animals are able to increase their anaerobic production of ATP by the simultaneous catabolism of carbohydrates and amino acids. A variety of end-products may result, including succinate, alanine, propionate, isobutyrate, and isovalerate (Hochachka 1980; Hochachka et

al. 1973; Kohler and Stahel 1972; Rao and Rao 1983; Saz 1981; Zebe et al. 1981; Zwaan and Wijsman 1976). The use of these alternative pathways has the advantages of generating more ATP and avoiding acid end-products when compared to classical anaerobic glycolysis. The use of such alternative anaerobic pathways by the brains of fish is not known; this study provides data concerning the generation of these endproducts in the brain during anoxia.

Rainbow trout (Salmo gairdneri) and brown bullhead catfish (Ictalurus nebulosus) occupy ecologically distinct habitats. The trout inhabits cold, fast-flowing waters while the bullhead is generally found in warm, still waters. These species exhibit large differences in tolerance to hypoxia and anoxia. Although the oxygen binding characteristics of the blood of these two species differs, the amount of hemoglobin, oxygen capacity, and plasma pH are comparable (Burton and Heath 1980; Haws and Goodnight 1962; Satchell 1971). The objectives of this study were to determine to what extent the differences in anoxia tolerance between the trout and bullhead catfish can be accounted for by: 1) the maintenance of an energy supply within the bullhead brain but not in the trout; 2) the storage of more fuel in the bullhead brain; 3) the use of classic anaerobic glycolysis and alternative pathways in the bullhead brain but not in the trout brain.

2. LITERATURE REVIEW

2.1 Information Concerning Fish Brains

In dealing with fish and reduced ambient oxygen levels, the majority of research has focused on muscle (Batty and Wardle 1979; Burton and Heath 1980; Burton and Spehar 1971; Driedzic and Hochachka 1975; Heath and Pritchard 1965; Johnston 1975a,b; Jorgensen and Mustafa 1980a,b; Shoubridge and Hochachka 1980; Smith and Heath 1980; Thillart and Kesbeke 1978; Thillart et al. 1982; Waarde et al. 1982; Wardle 1978), blood (Burggren and Cameron 1980; Caillouet 1968; Heath and Pritchard 1965; Holeton 1977; Jonas and Bilinski 1965; Soivio et al. 1980; Thomas and Hughes 1982; Weber and Lykkeboe 1978), and other organs and/or whole body changes (Burton and Heath 1980; Johnston 1975b; Johnston and Bernard 1983; Thillart et al. 1976; Waarde and Henegouwen 1982; Wokoma and Johnston 1983). Most experiments involving fish brains have dealt with deficit function, stimulation of various areas (Bernstein 1970), or with preliminary investigations of biochemical parameters (Ananichev 1961; Breer and Rahmann 1974; Ehrlich and Cserr 1978; Shaffi 1978,1980,1981,1982). The brain of fish performs functions basic to all vertebrates, namely input, command, and integration. The typical teleost brain (Figure 1) is similar to other vertebrates (Smith 1982) as is the ultrastructure of the

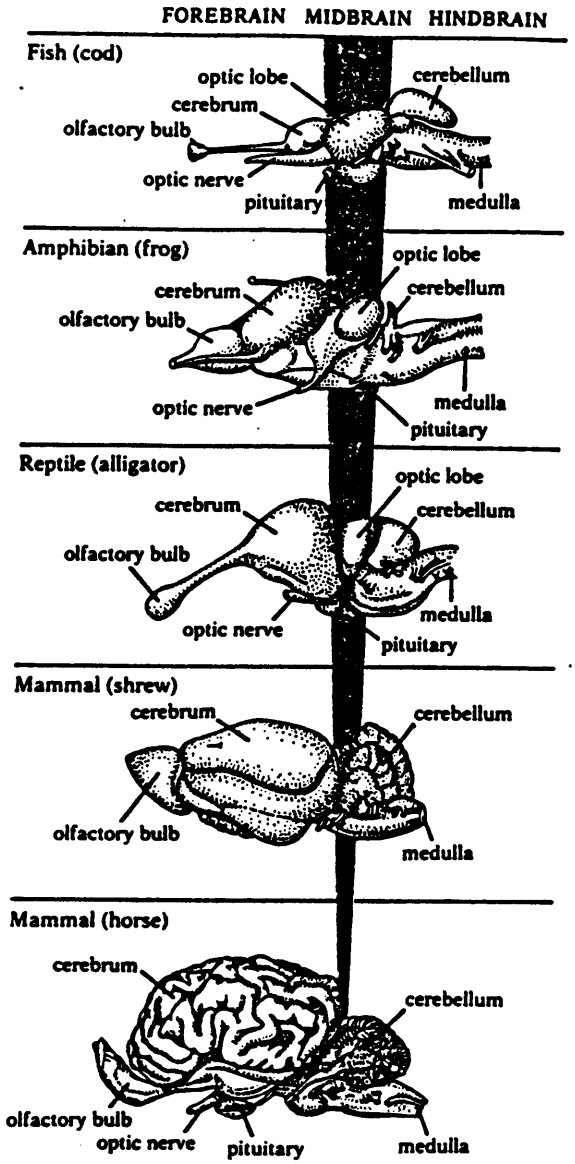


FIGURE 1. Comparison of vertebrate brains
(From Keeton 1976, pg. 455)

neuron (Palay et al. 1962). The brain occupies an anterodorsal position and is encased in a bony skull. Although the relative sizes of the brain divisions vary greatly in the different classes, the major subdivisions retain their association with important sense organs. The hind brain, or medulla oblongata, is the most strongly conserved in the vertebrate line. It is responsible for visceral functions which are basic to life (Marshall and Hughes 1980). The medulla, even when separated from the influence of the higher brain and spinal cord, is capable of coordinating rhythmic breathing movements (Shelton 1970). It would, therefore, be of paramount importance to the survival of the animal that the integrity of the brain, especially the medulla, be maintained during anoxia.

Studies on higher vertebrates have found that nervous tissue, compared to other tissues, is the least capable of withstanding oxygen deprivation. In mammals, the brain is one of the first areas to be affected by reduced oxygen (Hochachka 1980; Liere and Stickney 1963). The brain degenerates due to its high energy demand (Sick et al. 1984) and damage is irreversible (Bandurski et al. 1968). Animals have defense mechanisms to reduce or prevent energy loss from nervous tissue. Various studies with mammals have indicated that decreased oxygen causes increased cerebral blood flow (Liere and Stickney 1963) which is coupled to bradycardia and decreased peripheral blood flow (Scholander

et al. 1962). In fish, venous blood is pumped from the heart through the ventral aorta into the afferent branchial arteries of the gills, where gas exchange occurs. Blood leaves the gills by the efferent arteries and enters the brain via the carotid artery and the rest of the body by the dorsal aorta (Petterson and Nilsson 1980; Smith 1982). During normoxia, the brain receives highly oxygenated blood. Under the influence of hypoxia, dilation of brain capillaries can occur, increasing the surface area five-fold (Scheich et al. 1972). As will be discussed below, hypoxic fish exhibit bradycardia as well as changes in respiration and blood flow. McDougal et al. (1968) and Scheich et al. (1972) examined the effects of anoxia on fish brains, and several authors studied reptile brains (Belkin 1968; Bellamy and Petersen 1968; Lai and Miller 1973; Lutz et al. 1980; Robin et al. 1979; Sick et al. 1984). In these studies, it was found that animals tolerant of anoxia (e.g., goldfish and turtle) stored glycogen and CrP and were able to generate ATP by anaerobic glycolysis.

2.2 Responses of Trout and Bullhead Catfish to Hypoxia

The rainbow trout (Salmo gairdneri) and the brown bullhead catfish (Ictalurus nebulosus) occupy ecologically distinct habitats. The trout is an active fish found in waters with a high oxygen content. The bullhead is a

ubiquitous species, commonly found in warm waters where the oxygen content is low (Haws and Goodnight 1962; Satchell 1971; Saunders 1962). Trout and bullhead also exhibit large differences in tolerance to hypoxia and anoxia. Part of these tolerance differences may be explained by dissimilarities in blood properties (Burton and Heath 1980). Trout load and unload oxygen at a higher partial pressure of oxygen (Black 1955; Satchell 1971). The bullhead dissociation curve is to the left of the trout indicating that it is able to pick-up and release oxygen at a lower partial pressure of oxygen (Burton and Heath 1980; Grigg 1969; Haws and Goodnight 1962). Various researchers (Black 1955; Haws and Goodnight 1962; Saunders 1962) have found that catfish have a great resistance to external CO_2 levels while trout exhibit a large Bohr effect (Black 1955) which is a decrease in oxygen affinity caused by CO_2 (Riggs 1970). Although the oxygen binding characteristics of the blood from these two species differs, the amount of hemoglobin, oxygen capacity, and plasma pH are comparable (Haws and Goodnight 1962; Satchell 1971).

Trout and catfish exhibit some similar physiological responses upon exposure to hypoxia and anoxia. Both show bradycardia (Marvin and Heath 1968; Satchell 1971) which could reduce metabolic demand. Both species exhibit hyperventilation under mild hypoxia (Marvin and Heath 1968)

which could enable the fish to maintain a diffusion gradient favoring the removal of oxygen. At some level of hypoxia, the energy expended to increase respiration will be greater than the benefits derived and ventilation will decline. During hypoxia, both species should show increased branchial circulation resistance and increased peripheral vascular bed resistance (Satchell 1971). The former should facilitate the up-take of oxygen while the latter should help blood reach a critical area such as the brain.

Upon exposure to gradual hypoxia, bullheads are able to decrease their resting metabolism (Burton and Heath 1980; Grigg 1969). Marvin and Heath (1968) also noted decreased oxygen consumption by trout in water containing less than sixty percent oxygen saturation; catfish immediately reduced oxygen consumption at all levels below full saturation. Soivio et al. (1980) note that a quick response to hypoxia by trout is a decrease in blood pH. This decrease is most likely a metabolic acidosis due to increased lactic acid (Thomas and Hughes 1982). A similar phenomenon has also been reported in the channel catfish (Ictalurus punctatus) by Caillouet (1968) and Burggren and Cameron (1980). No data for the bullhead exist but a similar response is expected since blood lactate levels increase following anoxia. This reduction in blood pH results in decreased blood oxygen capacity (Caillouet 1968; Satchell 1971; Soivio et al. 1980) due to the Root effect

which predicts decreased oxygen capacity at acid pH (<6.5) (Riggs 1970).

2.3 Use of Proteins, Lipids, and Carbohydrates in Normoxia

Before any changes in the brain during anoxia can be understood, normal fish metabolism must be addressed. Unlike mammals, the primary diet of fish consists of protein, followed by lipids, and then carbohydrates (Love 1970). Those amino acids not necessary for growth have their amino group removed (Smith 1982), leading to the formation of ammonia as an excretory product and to the generation of carbon skeletons (Waarde 1983). The fate of the carbon skeletons from amino acids can be entry into either the tricarboxylic acid (TCA) cycle (as pyruvate, acetoacetyl-CoA, acetyl-CoA, alpha-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate) or they may also be converted into ketone bodies, glucose and glycogen, or lipids (Hochachka 1969; Lehninger 1982; Smith 1982; Waarde 1983). The capacity of fish to use deaminated protein as an energy source is great since under normal conditions they oxidize a large portion of the resulting carbon structures (Waarde 1983).

Lipids are a rountine energy source for fish (Love 1970; Smith 1982). Fatty acids of fish, usually 16 to 22 carbons long, undergo beta-oxidation, a process whereby the fatty acyl chain is shortened by one 2-carbon acetyl group

(as acetyl-CoA) at a time (Hochachka 1970; Lehninger 1982; Smith 1982). The hydrogen atoms removed from the fatty acid groups have their energy tapped through the electron transport chain (ETC) and the acetyl-CoA units enter the TCA cycle and are oxidized to CO_2 and water; therefore energy is generated in two processes when fatty acids are oxidized. In the liver, if too much acetyl-CoA is produced from fatty acids, formation of acetoacetate and beta-hydroxybutyrate (ie. ketone bodies) results. These compounds can be oxidized in other tissues (Lehninger 1982), or excess carbons from proteins, lipids, or carbohydrates can be stored as triglycerides (Smith 1982).

Carbohydrates obtained from the diet are a relatively unimportant energy source in predatory and scavenger species of fish. However, glucose and glycogen are available, being formed from proteins (Love 1970; Smith 1982). Glucose is catabolized to two molecules of pyruvate via glycolysis. Fish have been shown to possess the enzymes involved in glycolysis (Hochachka 1970; Knox et al. 1980; Walesby and Johnston 1980). In normoxic conditions, the majority of pyruvate is oxidized to acetyl-CoA and enters the TCA cycle. Some variable amount of pyruvate is reduced to lactate (Lehninger 1982).

2.4 Generation of High-Energy Phosphate Compounds in the Absence of Oxygen

During anoxia, the fish must produce ATP so that basic functions such as nerve transmission and muscle contraction can occur. Several studies have dealt with ATP levels during hypoxia or anoxia. In those involving muscle (white or red), ATP levels start at 3-10um/g and usually decrease non-significantly to 2-5um/g. Kidney and liver are not able to maintain their original levels of 1.5um/g, falling to less than 1um/g (Jorgensen and Mustafa 1980; Thillart et al. 1976, 1980). Shaffi (1980) reported values of 2-13um/g for the normoxic brains of six species of tropical catfish and carp. Initial values of ATP for goldfish, turtle, and frog brains were similar (1.1 to 1.9um/g) and decreased with anoxia by decapitation to 0.15 to 0.25um/g (McDougal et al. 1968). Belkin (1968) suggested that the failure of the central nervous system during anoxia in turtles, crocodiles, and snakes was due to lack of metabolic energy. McDougal et al. (1968) imply that ectotherms have a metabolic rate approximately ten times less than in mammalian brains (e.g., mouse). However, Robin et al. (1979) found no difference in basal aerobic metabolism in slices of turtle and rat brain. They do suggest that during anaerobic conditions, such as diving, the turtle is able to reduce its brain energy requirements; Sick et al. (1984) reached a similar conclusion. Hochachka (1985) has recently proposed that anoxia-tolerant species decrease their ATP demand; these species possess cell

membranes which are more impermeable to ions. It is not known if trout and catfish brain have different metabolic rates or membrane permeabilities.

In mammals, the brain depends almost exclusively on the catabolism of glucose for the production of ATP. The mammalian brain relies on a constant supply of glucose from the blood (Lehninger 1982). In fish, carbohydrates are the first to be affected during stress as they are readily utilized (Love 1970). It seems that fish use carbohydrates as their anaerobic substrate (Waarde 1983). Shaffi (1982) states that glucose is the main energy source of fish brains, and glycolysis is the major pathway of the CNS. Previous work by Shaffi (1981) showed that aerobic metabolism via sugar phosphates from glucose is the predominant form in the normoxic brain of fish. In normal pike, ling, and bream, Ananichev (1961) found that brain contained more glucose (by percent dry weight) than muscle or liver. In anoxic goldfish brain, the level of glucose fell from 5.3 to 1.0um/g (McDougal et al. 1968). Reptiles are well known for being able to tolerate anoxia and Belkin (1968) reported that anaerobic glycolysis maintained reptile CNS activity during such exposure. In two separate studies involving turtles, Robin et al. (1979) and Sick et al. (1984) concluded that in the brain, anaerobic glycolysis contributed significantly to ATP generation.

Brain tissue is not able to utilize lipids directly; instead it is able to use ketone bodies (B-hydroxybutyrate and acetoacetate). The acetyl groups formed in the liver are transported as ketone bodies to other parts of the body where they enter the TCA cycle and generate energy (Lehninger 1982; Smith et al. 1983). In fish, the few studies which examined levels of ketone bodies were conducted to determine the use of lipids during starvation (Hille 1982; Jonas and Bilinski 1965). One study by Thillart et al. (1982) reported ketone body values in tissues of goldfish after twelve hours of anoxia. Acetoacetate levels did not change with anoxia (red muscle-0.04, white muscle-0.02, liver-0.13, blood-0.03um/g) but B-hydroxybutyrate levels increased in red (0.11 to 0.16um/g) and white (0.14 to 0.21um/g) muscle and liver (0.03 to 0.07um/g); no change occurred in blood (0.07um/g). A reduction in flux through the TCA cycle was ruled out. Perhaps the accumulation of beta-hydroxybutyrate maintains redox balance as discussed by Hochachka (1980). The role of ketone bodies in the brains of fish is not known.

2.5 Storage Forms of Energy-Yielding Compounds

One way to increase anoxia tolerance is to increase the storage of compounds which can be used in the generation of ATP (Hochachka 1980). Creatine phosphate (CrP) is a storage form of high-energy phosphates and can

donate its phosphate group to ADP to form ATP (Lehninger 1982). Levels of ATP are buffered by CrP and will not fall until the concentration of CrP drops, indicating a depletion of energy stores (Jorgensen and Mustafa 1980b). There is variation among fish species in the amount of CrP in the muscle and liver tissues (for species and levels see: Johnston and Bernard 1983; Jorgensen and Mustafa 1980b; Thillart et al. 1976, 1980). Mammalian brains contain a ratio of two CrP to one ATP and deplete these stores when ATP is limited (Smith et al. 1983). McDougal et al. (1968) found initial levels of CrP in goldfish brain to be 3.9um/g, and these fell to 0.2um/g during anoxia. Turtle and frog brains had higher normoxic levels (5.6um/g) but similar anoxic ones. This gives fish an approximately three to one ratio of CrP to ATP; a seven to one ratio has been measured in lamprey nervous tissue (Rovainen et al. 1969).

Glycogen is the main storage form of glucose and is easily stored and mobilized (Drummond and Black 1960). Most glycogen is formed from protein, as little carbohydrate us converted to glycogen in fish (Waarde 1983). A glucose residue removed from a glycogen molecule is first converted to glucose-1-phosphate and then to glucose-6-phosphate for entry into the glycolytic pathway (Smith et al. 1983). Mammalian brains contain little glycogen, relying on transport of glucose by the blood (Lehninger 1982).

Glycogen values have been reported for many fish species, tissues, and conditions. In those studies subjecting the fish to hypoxia or anoxia, the following results were found: 1) carp whole body levels decreased from 40.5 to 24.5um/g (Johnston and Bernard 1983); 2) plaice liver levels decreased (64.2 to 17.9um/g) while muscle and heart did not (Jorgensen and Mustafa 1980a); 3) levels in red and white muscle of rainbow trout fell significantly (Johnston 1975b); 4) cutthroat trout and bluegill sunfish muscle and liver stores were greatly reduced (Heath and Pritchard 1965); 5) whole body levels in goldfish (9.5um/g) did not change (Thillart and Kesbeke 1978); 6) concentrations in goldfish liver and red muscle decreased (746 to 357um/g and 33 to 14um/g) while those of white muscle (13um/g) did not fall (Thillart et al. 1980); 7) levels decreased in the brains of goldfish (12.8 to 4um/g), turtle (19.3 to 4um/g) and frog (18.1 to 7um/g) with initial values being two to nine times higher than those found in mammalian brains (McDougal et al. 1968). Breer and Rahmann (1974) investigated the influence of temperature upon brain glycogen stores of goldfish and minnow. Their study found that brain levels increased with decreasing temperatures while liver levels remained constant. The values for goldfish brains were 26.2 to 20.0 to 13.1um/g as temperature increased from 7 to 12 to 20^{o}C. Minnow levels

decreased similarly, 20.0 to 11.5 to 4.2um/g.

2.6 Maintenance of Oxidation-Reduction Balance

To sustain anoxia, an organism must balance each oxidative step with a reductive one (Hochachka 1980). Nicotinamide adenine dinucleotide [phosphate] (NAD)[P] is frequently employed as a coenzyme of dehydrogenases; it accepts electrons (H^- or hydride ion) from a substrate. This reduced form (NADH or NADPH) is reoxidized by transfer of its electrons to an acceptor, leading to ATP, metabolite, or fatty acid synthesis (Lehninger 1982). During anoxia, the ability to transfer electrons should be reduced since oxygen is the final electron acceptor. This will affect reversible reactions and generation of ATP. Direct measurement of these nucleotides will not differentiate between free and bound forms nor between cytoplasmic and mitochondrial pools (Williamson et al. 1967). Use of a substrate ratio procedure, where the ratio of NAD^+ to NADH is calculated from the measured values of oxidized and reduced substrates such as pyruvate / lactate and acetoacetate / B-hydroxybutyrate, has been suggested. The lactate dehydrogenase system reflects the cytoplasm ratio while the B-hydroxybutyrate dehydrogenase reflects that in the mitochondria (Krebs 1967; Lai and Miller 1973; Thillart et al. 1982). There are several problems with these calculations. The major one deals with the assumption

that tissue pH remains constant (Thillart et al. 1982). Since lactate will accumulate in the tissue, a constant pH cannot be assumed. As no data exist as to reasonable values for trout or catfish brains either during normoxia or anoxia, such calculations will not be attempted. Instead, the various alternative endproducts and their proposed pathways will give a better clue to the redox state of the brains during anoxia (see Hochachka 1980; Hochachka and Mommsen 1983; Hochachka and Mustafa 1972; Hochachka et al. 1973).

2.7 Classic Metabolism Under Anaerobic Conditions

Lactate is the end-product of classical anaerobic glycolysis. When fish are subjected to anoxia, they appear to use anaerobic metabolism as a supplement or replacement mode of energy release and lactic acid accumulates (Burton and Heath 1980; Driedzic and Hochachka 1975; Heath and Pritchard 1965; Johnston 1975a,b; Smith and Heath 1980; Thillart and Kesbeke 1978). Under anoxic conditions, the two molecules of pyruvate formed from glucose can be reduced to two molecules of lactate by lactate dehydrogenase. In this pathway, the oxidative step of glycolysis (GA3P to 1,3BPG) is balanced by a reductive one (Pyr to Lac). There is a net production of two ATPs via substrate-level phosphorylation. Lactate contains much of the energy of glucose and this can be released upon

oxidation to CO_2 and water when oxygen is available. During anoxia the formation of lactate is useful in releasing some of the free energy of glucose without oxidizing it (Hochachka 1969, 1980; Lehninger 1982). Pyruvate has been measured in red and white muscle (Burton and Heath 1980; Burton and Spehar 1971; Johnston 1975a,b; Smith and Heath 1980; Thillart et al. 1982), blood (Hille 1982; Thillart et al. 1982), liver (Burton and Heath 1980; Burton and Spehar 1971; Johnston 1975a), and brain (Shaffi 1978) of different fish species. No clear trend emerges from these studies as in most cases pyruvate does not change, while in a few others it either increases or decreases with hypoxia or anoxia. The accumulation of lactate has been extensively measured in fish following hypoxia or anoxia: 1) goldfish muscle had increased levels (1.5 to 4um/g as mean values) in some studies (Thillart 1982; Thillart and Kesbeke 1978; Thillart et al. 1982) while other studies show no significant increase (Thillart et al. 1976, 1980); Thillart et al. (1980 and 1982) show increased liver (3.5 to 7um/g) and blood (2 to 7um/g) levels; 2) carp tissue levels increase (muscle 8.5 to 25um/g, body 3.5 to 8.4um/g, and liver 3.3 to 9um/g) (Driedzic and Hochachka 1975; Johnston 1975a; Johnston and Bernard 1983; Smith and Heath 1980); 3) plaice blood, liver, and muscle levels increase while heart and kidney do not (Jorgensen and Mustafa 1980a); 4) rainbow trout show increases in whole body (7.9 to 25um/g), muscle

(107 to 243mg/100g and 14 to 25um/g), liver (14 to 80mg/100g), and blood (1 to 7um/g) (Burton and Heath 1980; Burton and Spehar 1971; Johnston 1975b; Smith and Heath 1980; Thomas and Hughes 1982; Turner et al. 1983; Wokoma and Johnston 1983); 5) brown bullhead liver (3.6 to 104mg per 100g) and muscle (37 to 91mg per 100g) increased (Burton and Heath 1980). Accumulation of lactate has been reported in molluscs (Rao and Rao 1983; Zwaan and Wijsman 1976), turtles (Hochachka et al. 1975), trematodes (Kohler and Stahel 1972), and leeches (Zebe et al. 1981). McDougal et al. (1968) reported an increase in brain lactate levels following anoxia in goldfish (4.9 to 38um/g) and frog (2.9 to 34um/g).

2.8 Use of Modified Anaerobic Pathways

In animals able to withstand anoxia for extended periods of time, anaerobic glycolysis as described above would not supply enough energy (Hochachka 1980; Hochachka et al. 1973). Through the use of "modified" pathways, additional energy can be generated by coupling carbohydrate and amino acid catabolism with a number of endproducts being formed (Fields 1983; Hochachka and Mustafa 1972; Hochachka et al. 1973, 1975). Succinate is a proposed endproduct of amino acid catabolism. Two pathways to succinate are possible (see Figure 2). The first pathway involves the reaction of glutamate and pyruvate to yield

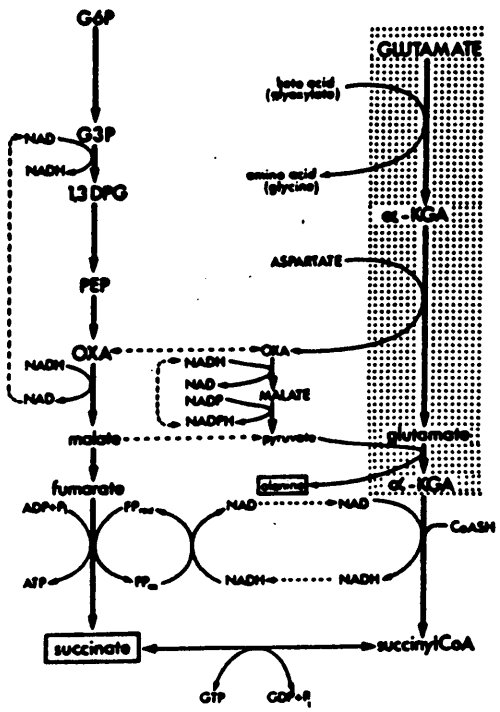


FIGURE 2. Pathway to succinate and alanine formation (From Hochachka et al. 1973, pg. 545)

Abbreviations used: G6P, glucose-6-phosphate; G3P, glyceraldehyde-3-phosphate; 1,3 DPG, 1,3 diphosphoglycerate; PEP, phosphoenol-pyruvate; OXA, oxaloacetate; α-KGA, α-ketoglutarate; NAD and NADH, oxidized and reduced nicotinamide adenine dinucleotide; NADP and NAD PH, oxidized and reduced nicotinamide adenine dinucleotide phosphate; FP_{ox} & FP_{red}, oxidized and reduced flavoprotein; ADP and ATP, adenosine di- and triphosphate; GDP and GTP, guanosine di- and triphosphate; P_i and PP_i, inorganic phosphate and pyrophosphate.

alpha-ketoglutarate and alanine (also considered to be an ananaerobic endproduct resulting from amino acid catabolism). The a-ketoglutarate enters the TCA cycle and is converted to succinate. The second pathway starts with aspartate conversion to oxaloacetate which is then reduced to malate. Malate is converted first to fumarate and then to succinate (by "fumarate reductase"; see Fields (1983) for discussion). Redox balance can be maintained by coupling these two pathways so that the first supplies reducing equivalents to the second; this will lead to the generation of seven moles of ATP per mole of glucose and two moles of aspartate and a-ketoglutarate (Hochachka et al. 1973). It has also been suggested that PEP can be converted to oxaloacetate for entry into the second pathway (Fields 1983).

Succinate, and to a lesser extent alanine, increases in molluscs (Rao and Rao 1983), trematodes (Kohler and Stahel 1972) and leeches (Zebe et al. 1982). Hochachka et al. (1975) found increases in these compounds in diving vertebrates. Fish have also been studied for increases following anoxia. Studies involving succinate have shown: 1) only plaice liver had increased levels (0.3 to 1.2um/g) while muscle, heart, and kidney levels were less than 0.3um/g (Jorgensen and Mustafa 1980a); 2) certain genera of carp showed increases (Johnston 1975a; Johnston and Bernard 1983) while others had decreases (Smith and Heath 1980) or

no change (Driedzic and Hochachka 1975) in muscle and whole- body levels; 3) goldfish red and white muscle (0.23 to 0.74 and 0.14 to 0.67um/g), blood (0.09 to 0.56um/g), and liver (0.44 to 0.75um/g) levels increase (Thillart 1982); 4) rainbow trout red and white muscle increased (1.8 to 7 and 1.1 to 3.3um/g in Johnston 1975b; 0.4 to 0.8 and 0.4 to 0.5um/g in Smith and Heath 1980).

Alanine has been measured in fish: 1) plaice muscle and heart levels increased (1.4 to 2.4um/g and 0.7 to 3.0um/g) while liver and kidney were unchanged (Jorgensen and Mustafa 1980a); 2) levels in carp muscle and body increased (Driedzic and Hochachka 1975; Johnston 1975a; Johnston and Bernard 1983; Smith and Heath 1980) with one reported decrease in red muscle of mirror carp (Smith and Heath 1980); 3) goldfish had increased levels in red (1.5 to 2.7um/g) and white (2.8 to 4.0um/g) muscle, liver (1.7 to 3.8um/g), blood (0.4 to 0.9um/g), and body (0.5 to 1.8um/g) (Thillart 1982; Thillart et al. 1976; Waarde et al. 1982); 4) rainbow trout red and white muscle increased (1.2 to 2.2 and 0.9 to 3.2um/g in Johnston 1975b; 4.2 to 8.0 and 3.7 to 6.7um/g in Smith and Heath 1980). Hochachka et al. (1973) has suggested that the generation of a-ketoglutarate and not the production of alanine is the significant point of this pathway. It appears that certain species (and only in some tissues) may be able to generate

energy by alternative pathways where anaerobic glycolysis and amino acid catabolism are coupled and succinate and alanine accumulate. The use of alternative pathways in the brains of fish is not known.

The formation of propionate from succinate occurs in certain bivalves (Zwaan and Wijsman 1976), leeches (Zebe et al. 1981), trematodes (Kohler and Stahel 1972) and ascarids (Saz 1981) during anoxia. This pathway can generate up to eleven ATPs per mole glucose and two moles of aspartate and a-ketoglutarate (Figure 3). Proprionate has not been shown to accumulate in fish (Driedzic and Hochachka 1975; Thillart et al. 1976); white muscle decreased from 0.5 to 0.1um/g in goldfish (Thillart et al. 1976). Isobutyrate and isovalerate have been detected in some helminths (see Figure 3) (Hochachka et al. 1973). Driedzic and Hochachka (1975) found no significant accumulation in carp white muscle. Data concerning propionate, isobutyrate, and/or isovalerate accumulation in nervous tissue are not available.

The use of these alternative pathways has the advantages of producing more ATP than anaerobic glycolysis and avoiding the generation of large quantities of an acid endproduct. Shoubridge and Hochachka (1980) have proposed an alternative to coping with lactic acid. In their study with anoxic goldfish, results suggest that lactate which is produced in muscle and liver is metabolized to ethanol

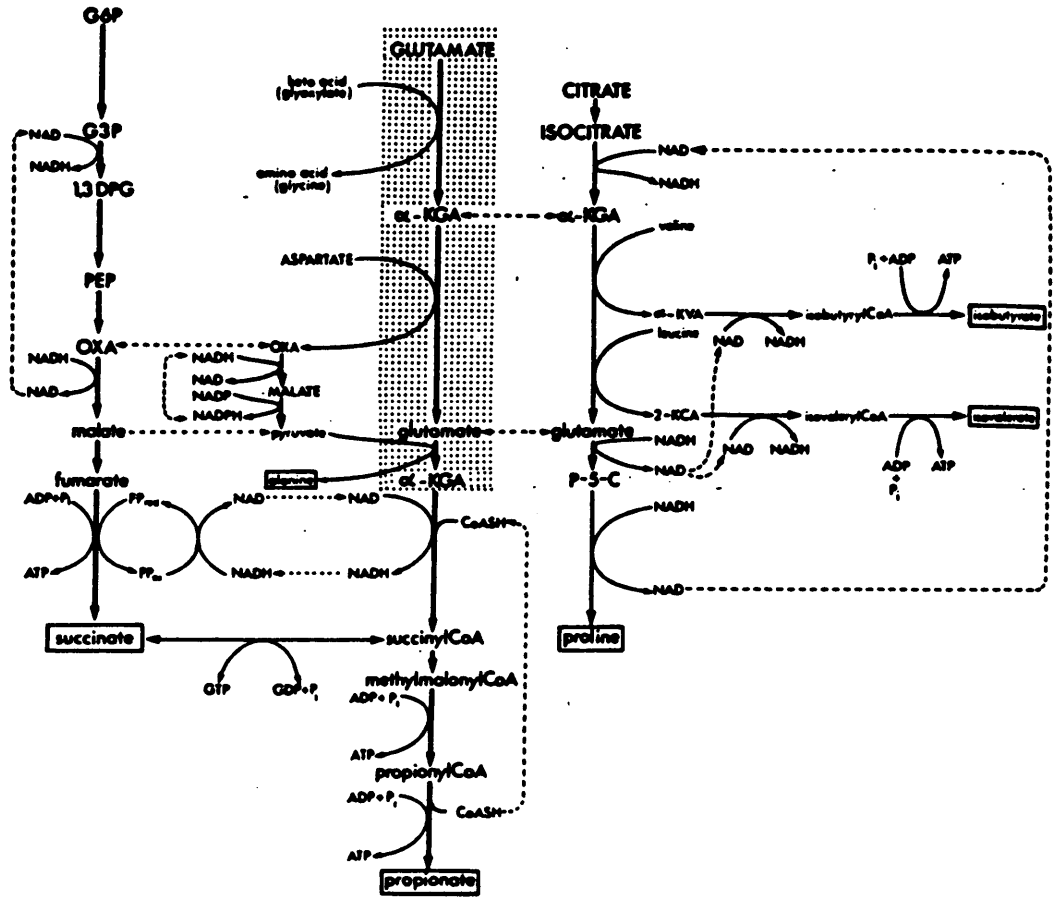
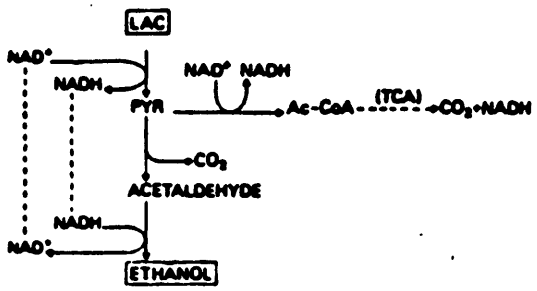


FIGURE 3. Pathway to propionate, isobutyrate, and isovalerate formation
(From Hochachka et al. 1973, pg. 550)

which can be excreted. Muscle and liver levels increased from non-detectable to 3.3um/g and 3.0um/g with 6.6um/g excreted into the water. It was also proposed that lactate produced in heart and brain is metabolized to ethanol in the muscle. This system allows for the production of a neutral endproduct and for easy removal of that product. Mourik et al. (1982) suggest that acetaldehyde serves as an electron acceptor to produce ethanol in goldfish muscle. Thillart et al. (1983) report a large difference between the amount of glycogen available in goldfish and the quantity of ethanol produced; they suggest protein as a carbon source for ethanol production. Johnston and Bernard (1983) found ethanol accumulating in Crucian carp following anoxia. The whole body level was 3.1um/g; liver was 2.2um/g; red muscle was 7.1um/g; white muscle was 3.9um/g. They accounted for ethanol from glycogen, noting that ethanol production does not generate ATP but allows for redox balance and no acid endproduct accumulation (Figure 4). It is not known if trout or catfish are able to produce ethanol either in the brain or other parts of the body.

a.



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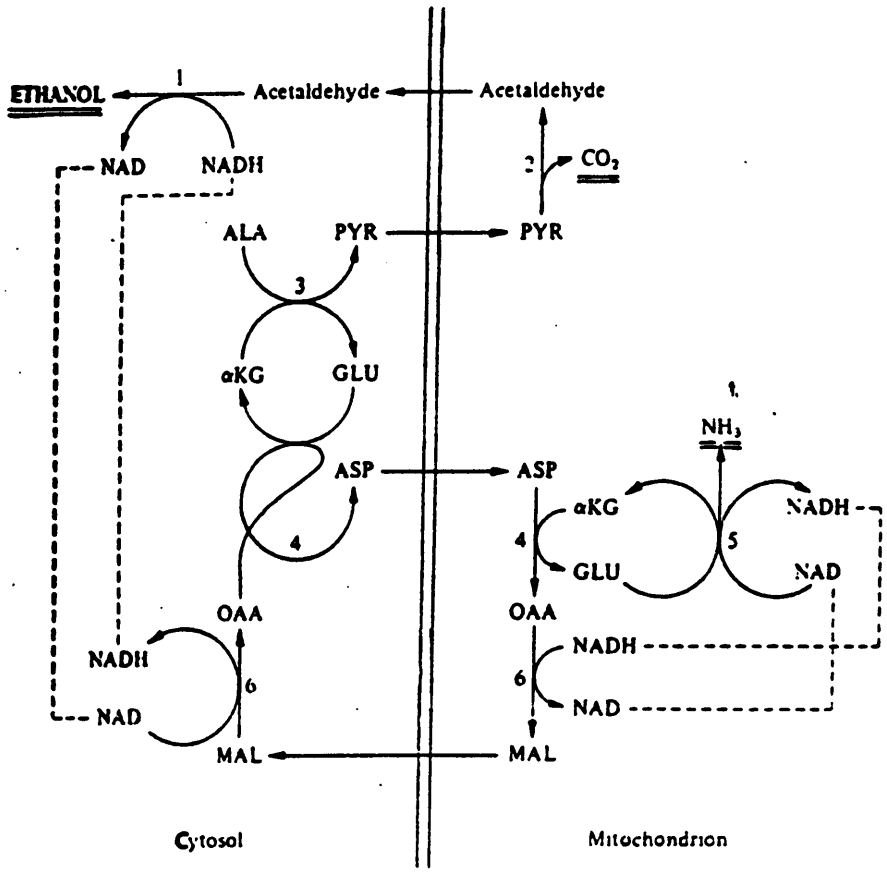

FIGURE 4. Pathways to ethanol formation
(a. From Thillart 1982, pg. 53)
(b. From Waarde et al. 1982, pg. 57)

3. MATERIALS AND METHODS

3.1 Experimental Animals

Rainbow trout (Salmo gairdneri) were obtained from the Wytheville (Virginia) State Fish Hatchery. They were maintained in a 390-liter tank in the laboratory which was supplied with flow-through, well-aerated, dechlorinated Blacksburg tapwater. Temperature was maintained at 15±1°C.

Brown bullhead catfish (Ictalurus nebulosus) were netted from Gaithright reservoir in Bath County, Virginia. To reduce injury, three to five millimeters of the dorsal and pectoral spines were removed with bone cutters. Bullheads were held in a manner similar to the trout except the temperature was maintained at 20±1°C. A smaller tank was kept at the test temperature of 25±1°C; catfish were acclimated in this tank for at least two weeks before use. The large holding tank was dosed biweekly, alternately, with acriflavin (15mg/l) or oxytetracycline (10mg/l) as a prophylactic measure since the bullheads suffered skin lesions during their capture. Trout were not treated as no damage occurred during their handling.

All fish were maintained in a 14L:10D photoperiod. On alternate days, animals were fed Purina trout chow ad libitum. All experiments were conducted at least 24 hours after feeding. Pertinent physical data is summarized in Table 1.

TABLE 1. Summary of trout and catfish physical data

	Brain Weight (g)	Body Weight (g)	Brain / Body (%)	Body Length (cm)
Trout				
Control (n=11)	0.31[a] ±0.02	163.42 ±11.11	0.190	25.07 ±0.89
Anoxic (n=12)	0.34 ±0.02	145.37 ±12.29	0.233	23.94 ±0.73
Catfish				
Control (n=10)	0.28 ±0.01	136.01 ±16.03	0.205	22.52 ±0.82
Anoxic (n=10)	0.33 ±0.01	158.35 ±10.48	0.207	22.83 ±0.48

[a]Mean ± S.E.M.

3.2 Test Conditions

The test chamber consisted of a plexiglass tube whose dimensions are given in Figure 5. A #15 rubber stopper was caulked into one end and a tube was inserted through the stopper; one end was connected to an air stone and the other to either a nitrogen or an air source. To produce an anoxic condition (<0.5ppm O_2), the contents of the tube (approximately 2300 milliliters of "home" tank water) were vigorously bubbled with nitrogen for twenty minutes. The oxygen concentration was checked with a Yellow Springs Instruments Model 51A oxygen electrode. The fish were netted, placed in a bucket of water, and transferred by hand into the chamber which was then sealed with a second #15 rubber stopper. Control animals were handled in a similar manner except the water was aerated throughout their stay in the tube.

The acclimation temperature was maintained by flowing water from the "home" tank through a wooden box which held the plexiglass tube. Aquarium heaters were used to maintain 25°C for the catfish. Temperature was monitored with a mercury thermometer throughout the experiment.

The desired endpoint was death from anoxia. With trout, the animals exhibited tetanic spasms of their operculae and tails before death. They were removed from the chamber when these spasms slowed. Catfish did not exhibit these tremors and often had periods of apnea so

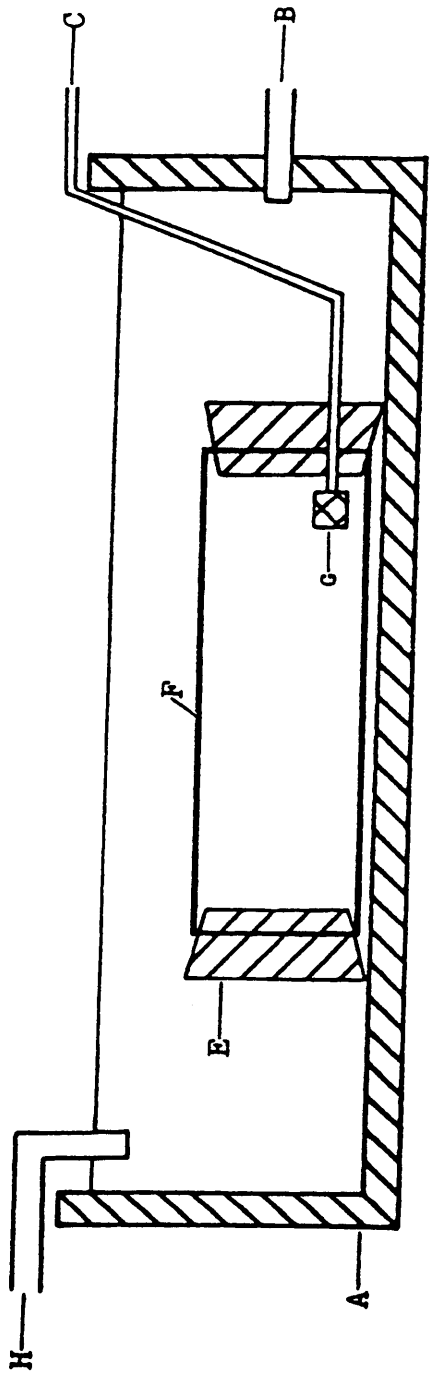

FIGURE 1. Experimental chamber and water bath set-up

A: Water bath (64.8cmL x 16.5cmW x 18.5cmH)
B: Water inlet
C: Air/nitrogen source
D: Water
E: #15 rubber stopper
F: Plexiglass chamber (27.5cmL x 9.4cm^{2}D)
G: Air stone
H: Water outlet

determination of death was more difficult. Catfish were removed from the test chamber when more than one minute elapsed between operular movements (Burton and Spehar 1971). Control fish were kept in the tube for approximately the same length of time as experimental ones. Time to death for all groups is given in Table 2.

3.3 Tissue Preparation and Analyses

Upon removal from the chamber, control animals were stunned with a blow to the head. All animals then had their spinal cord cut and the top of the skull quickly removed. The brain was pulled from the skull with forceps and placed in ice-cold 6% perchloric acid. This required approximately one minute or less. The brain was minced with scissors, weighed, and the volume of acid adjusted to five times the tissue weight. Blood samples of 0.2ml were pipetted from the ventral aorta and placed in 1ml 6% perchloric acid. The cells were then disrupted with a blunt glass rod. The brain mince was homogenized with a motorized Teflon pestle. All samples were spun at 10,000rpm for 15 minutes in a refrigerated centrifuge. For glycogen and alanine determination, 0.3ml aliquot was removed and refrigerated. The remaining supernatant was decanted, neutralized with 2.5M K_2CO_3, and spun at 3,000g for two minutes. The resulting supernatant was evenly distributed to two test tubes. One was used for ATP, CrP, and glucose

TABLE 2. Mean time to death(±S.E.M.) for trout and catfish

	Control (min)	Anoxic (min)
Trout	12.7[1a] ±1.5	12.0[a] ±1.3
Catfish	72.7[b] ±8.9	62.2[b] ±5.6

[1]Groups denoted by the same letter not significantly different at the p=0.05 level

determination; the other was diluted to twice its volume with ethyl ether and centrifuged at 3,000g for two minutes. The aqueous bottom layer was removed and acidified with 17.5% perchloric acid. This was filtered through a 0.45um filter and frozen at -70°C until analyzed on a high pressure liquid chromatograph (HPLC). Enzymatic assays were conducted within 24 hours after the sample was obtained; HPLC determinations were made 2 to 10 weeks after sampling. Assays for ethanol were conducted on brains which were deproteinated in 6.25% trichloroacetic acid; all other steps were as described above.

Four enzymatic assays were conducted using a Bausch and Lomb Spectronic 88 at 340nm. Alanine was measured by the method of Grassl (1974) in which the decrease in NADH is monitored. Glycogen (umoles glucosyl units per gram tissue wet weight) and glucose were determined by the Keppler and Decker method (1974). An increase in NADPH is proportional to the amount of glucose liberated from glycogen hydrolysis. ATP was determined in the manner described by Jaworek et al. (1974). Phosphocreatine was determined in the same tube as the ATP assay by the addition of ADP, creatine kinase, and glutathione (Walesby and Johnston 1980). Ethanol was determined using a Sigma diagnostic kit (Alcohol, ethyl: No. 332-UV) which is based on the reduction of NAD to NADH.

Eight anaerobic endproducts and two ketone bodies were

measured from a single 20ul sample using a Varian Model 500 HPLC. A Biorad Aminex HPX-87X ion exclusion column was used for sample separation. The best separation was achieved at 38°C using 5% acetonitrile in 0.013N sulfuric acid as the solvent (Guerrant et al. 1982). Column temperature was maintained with a water jacket. Detection of endproducts was with a Varian UV moniter at 214nm and the detector signal was integrated on a Hewlett-Packard integrator. Each sample peak was compared by retention time and area to the corresponding reference peak and the concentration automatically calculated. Table 3 lists the components of the standard solution, their concentrations, retention times, and source. Figure 6 shows the type of chromatograph obtained for a sample.

Data were analyzed for significant differences using a modified Duncan's multiple-range test. Comparisons were at the $p=0.05$ level.

TABLE 3. The standard HPLC solution and related information

Component	um/g	RT[1]	Source	#[2]
a-Ketoglutarate	5.0	5.9	Sigma	K 1750
Pyruvate	5.0	6.9	Sigma	P 2256
Succinate	5.0	7.9	Sigma	S 7501
Lactate	5.0	9.0	Sigma	L 2250
B-Hydroxybutyrate	5.0	9.8	Sigma	H 6501
Acetoacetate	5.0	10.1	Sigma	A 8509
Acetate	10.0	10.8	Fisher	A38-500
Propionate	10.0	12.7	Gift[3]	
Isobutyrate	21.5	14.1	Gift	
Isovalerate	10.0	16.1	Gift	

[1]Retention time in minutes
[2]Catalog number
[3]Gift from Dr. Smibert's laboratory

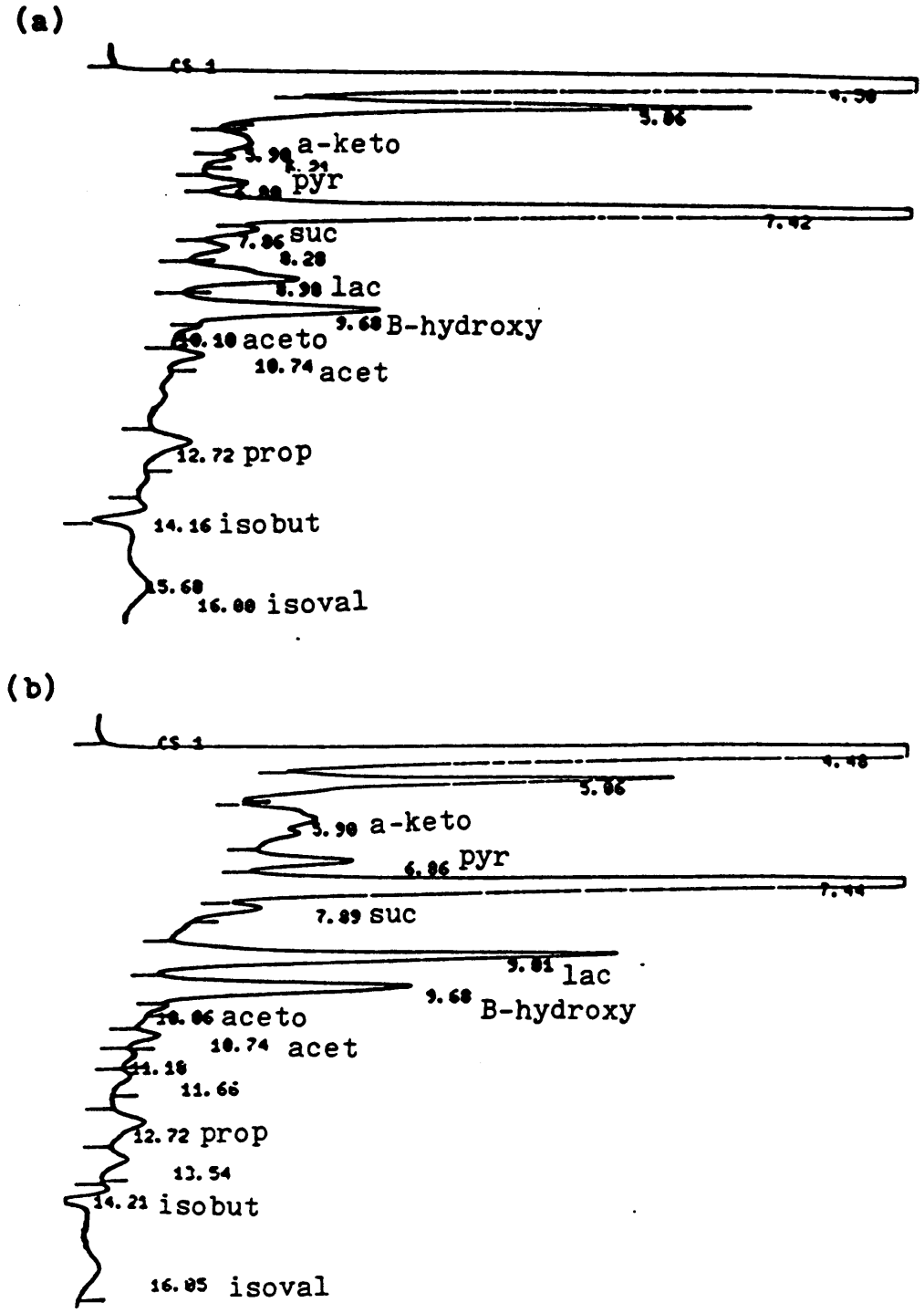

FIGURE 6. Chromatographs of trout brain samples (a)Trout control brain tracing. (b)Trout experimental brain tracing. Numbers refer to peak retention times in minutes.

4. RESULTS

4.1 Response to Anoxic Water

Although trout and catfish had different times to death in anoxic water, the two species exhibited similar behavior in the chamber. Upon exposure to anoxia, the animals became irritable as they attempted to escape the area. After this initial period of tail-thrashing, the fish became quiescent; both species appeared to increase the amplitude and frequency of their opercular movements. Approximately half-way through their exposure, the animals thrashed their tails and some turned around in the chamber. At this point, trout lost equilibrium, resting on the bottom until removed from the tube when ventilation ceased. Catfish usually remained upright through their exposure. They frequently attempted to leave the chamber even in the final minutes of their stay. The bullheads exhibited periods of apnea and were removed when more than sixty seconds elapsed between opercular movements.

4.2 Indicators of High-Energy Phosphate Generation

In trout, (Figure 7) there was no significant decrease in ATP levels following exposure to anoxia (0.752$\pm$0.101um/g for controls to 0.682$\pm$0.096 for anoxic brains). Catfish, however, had a significant reduction in the amount of ATP present at death. Average brain ATP levels dropped 40% from normoxic values of 1.621um/g to anoxic values of 0.959um/g.

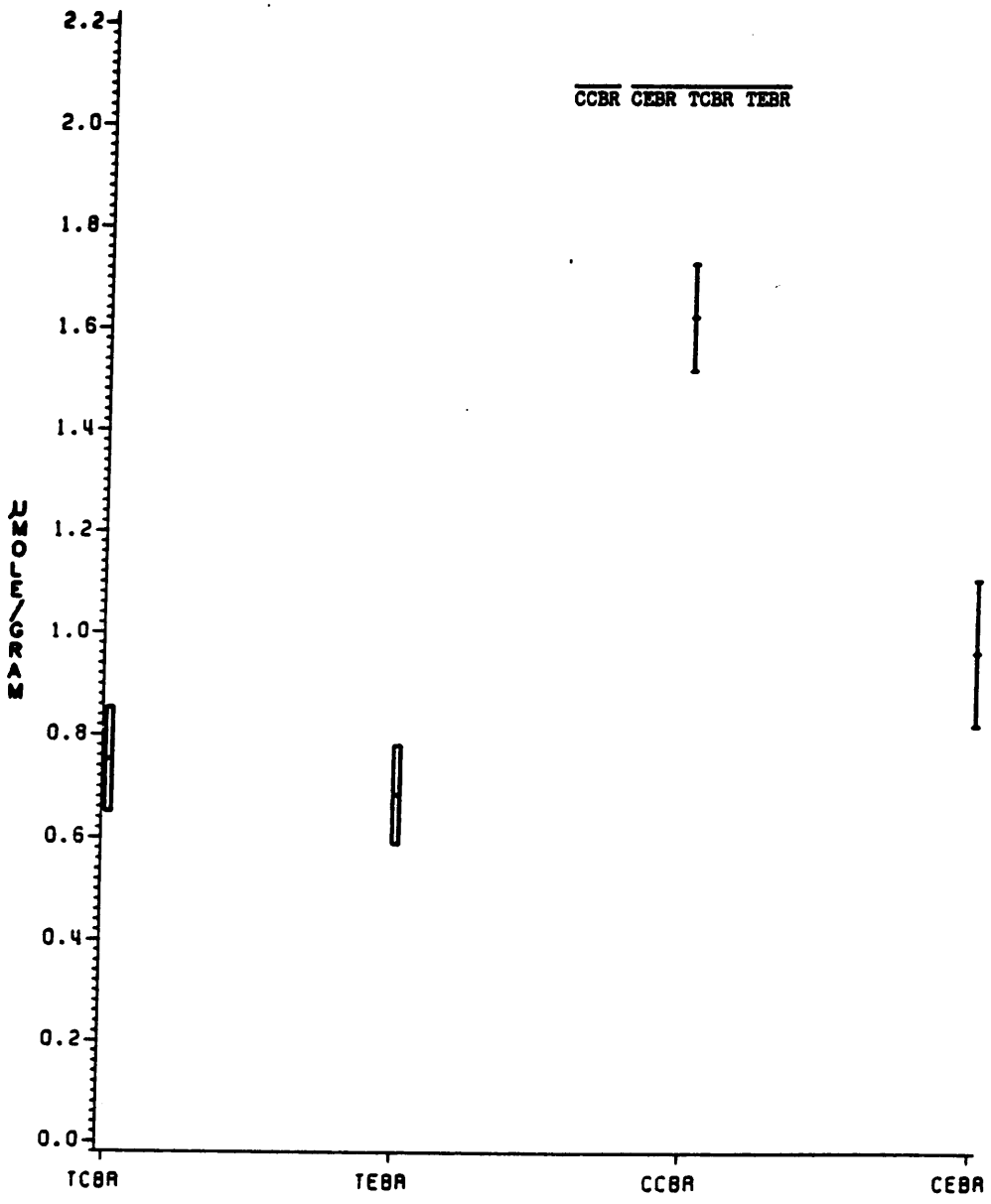

FIGURE 7. Adenosine triphosphate (Mean±1S.E.M.) TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; Groups under the same line are not significantly different at the p=0.05 level. N=9-12.

Control catfish brains had approximately 100% more ATP than control trout brains.

Neither species exhibited any change in glucose concentrations following anoxia (Figure 8). There was however a significant difference between the species; control catfish possessed four times more free glucose than control trout.

The concentrations of beta-hydroxybutyrate and acetoacetate were determined for brain and blood of trout and bullhead. Beta-hydroxybutyrate levels (Figure 9) in anoxic trout brains (14.31um/g) increased to 184% of normoxic levels (7.769um/g), with the blood exhibiting a similar, but non-significant, increase. The anoxic trout brain had a higher concentration of B-hydroxybutyrate than its blood while normoxic brain and blood were comparable. Levels of B-hydroxybutyrate in the catfish brain were not significantly decreased following anoxia. As with the trout, concentration in the catfish blood appeared to be lower than in the brain. The second ketone body, acetoacetate (Figure 10), displayed similar patterns in both species, namely that anoxic brain and blood values were higher than their respective control values. Brain levels tended to be higher than the corresponding blood levels.

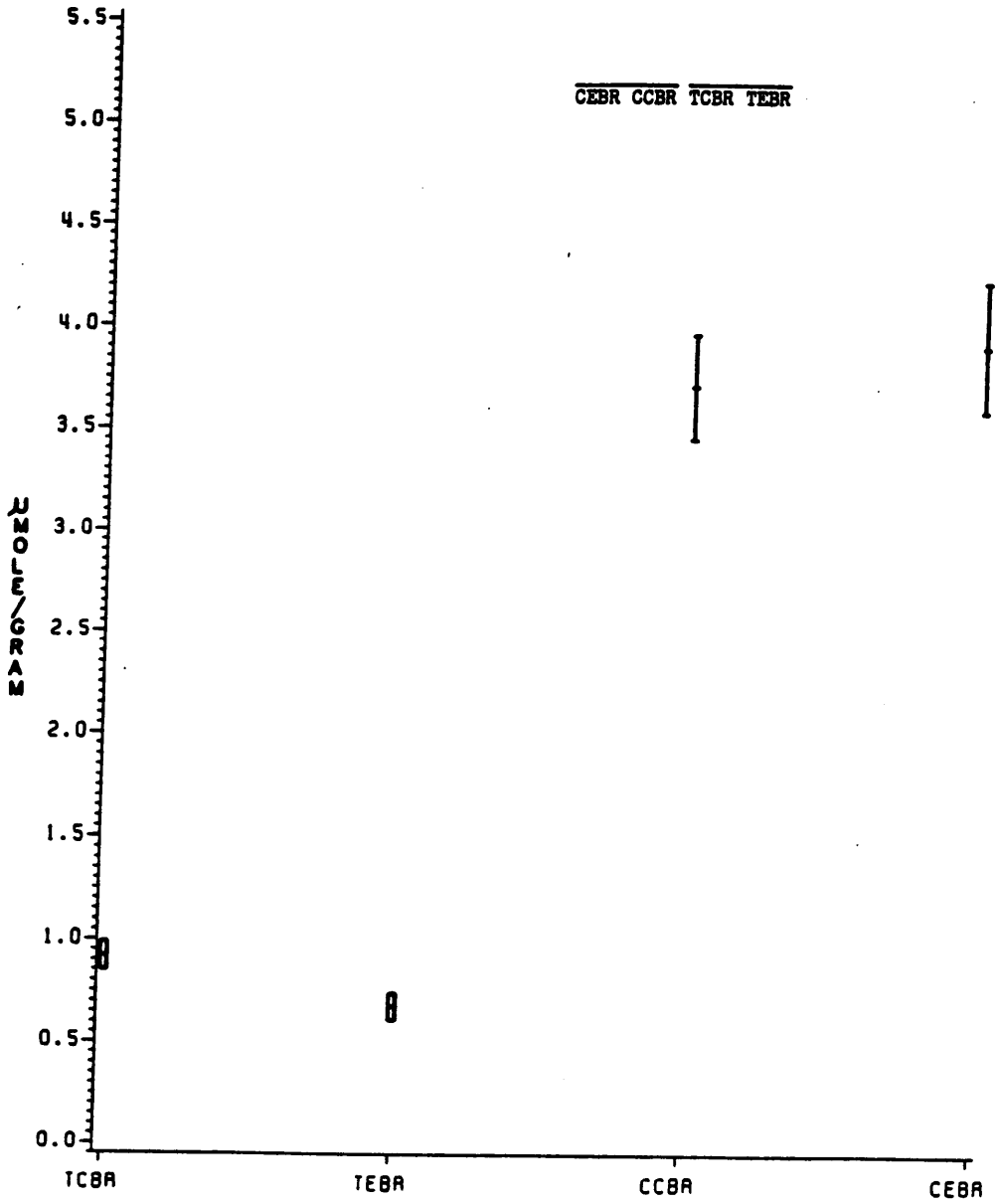

FIGURE 8. Free glucose (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain;
CCBR=Catfish control brain;CEBR=Catfish experimental brain;
Groups under the same line are not significantly different
at the p=0.05 level. N=10-11.

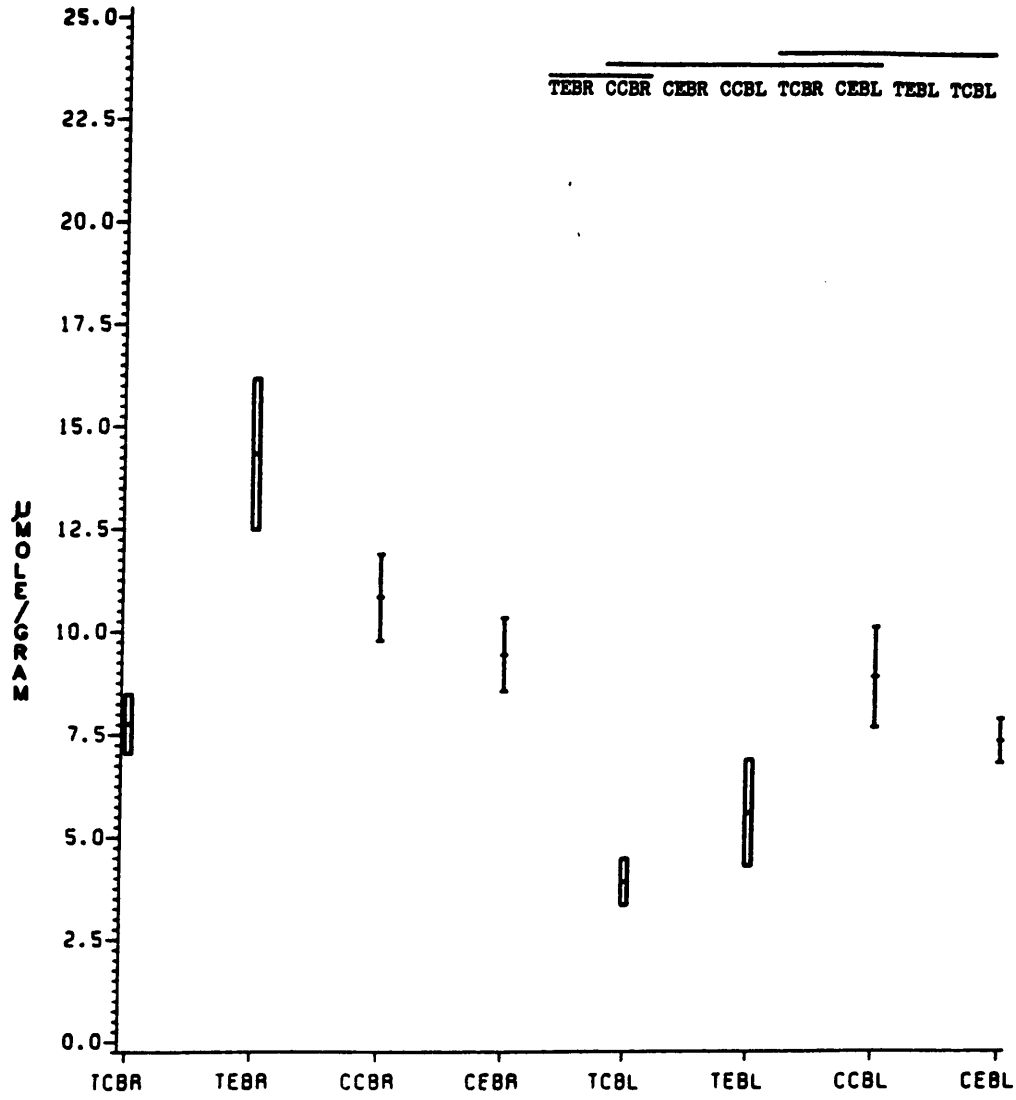

FIGURE 9. Beta-hydroxybutyrate (Mean±1S.E.M.) TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; TCBL=Trout control blood; TEBL=Trout experimental blood; CCBL=Catfish control blood;CEBL=Catfish experimental blood; Groups under the same line are not significantly different at the p=0.05 level. N=9-11 for brain and N=5 for blood.

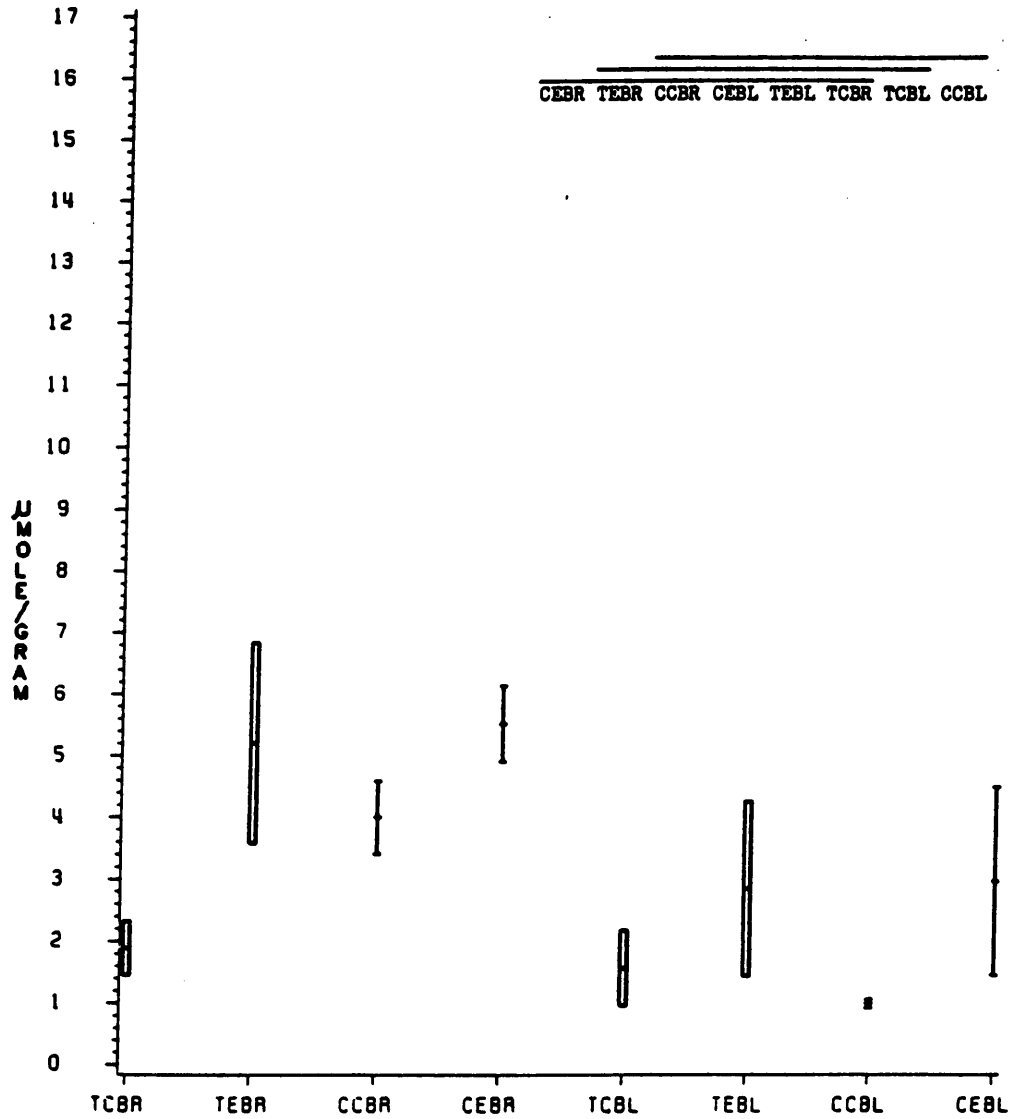

FIGURE 10. Acetoacetate (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; TCBL=Trout control blood; TEBL=Trout experimental blood; CCBL=Catfish control blood;CEBL=Catfish experimental blood; Groups under the same line are not significantly different at the p=0.05 level. N=9-10 for brain and N=3-5 for blood.

4.3 Storage of Energy-Yielding Compounds

A major storage form of high-energy phosphate is creatine phosphate. In anoxic trout brains there was a non-significant reduction in phosphocreatine levels following exposure to anoxia (Figure 11). Bullheads, however, showed a dramatic decrease of approximately 80% following anoxia (0.806um/g to 0.155um/g). Normoxic catfish brains stored 147% more creatine phosphate than normoxic trout brains (0.806um/g compared to 0.326um/g).

Trout and catfish brains exhibited significant decrease in glycogen content (Figure 12). Trout had a 56% reduction, going from a normoxic value of 2.844um/g to an anoxic level of 1.254um/g. Catfish brain glycogen declined 61% (15.85um/g for controls to 6.15um/g for anoxia exposed). Brains of control bullhead stored nearly six times more glycogen than comparable trout brains.

4.4 Indicators of Anaerobic Metabolism

Levels of lactate in anoxic trout brain and blood (11.76um/g and 4.577um/g) increased significantly over their respective control levels (5.443um/g and 2.894um/g) (Figure 13). Catfish brain and blood had similar increases: following anoxia, brain concentrations rose from 10.04um/g to 20.08um/g and blood concentrations rose from 5.000um/g to 9.933um/g. Both species had higher levels of lactate in their brains than in their blood.

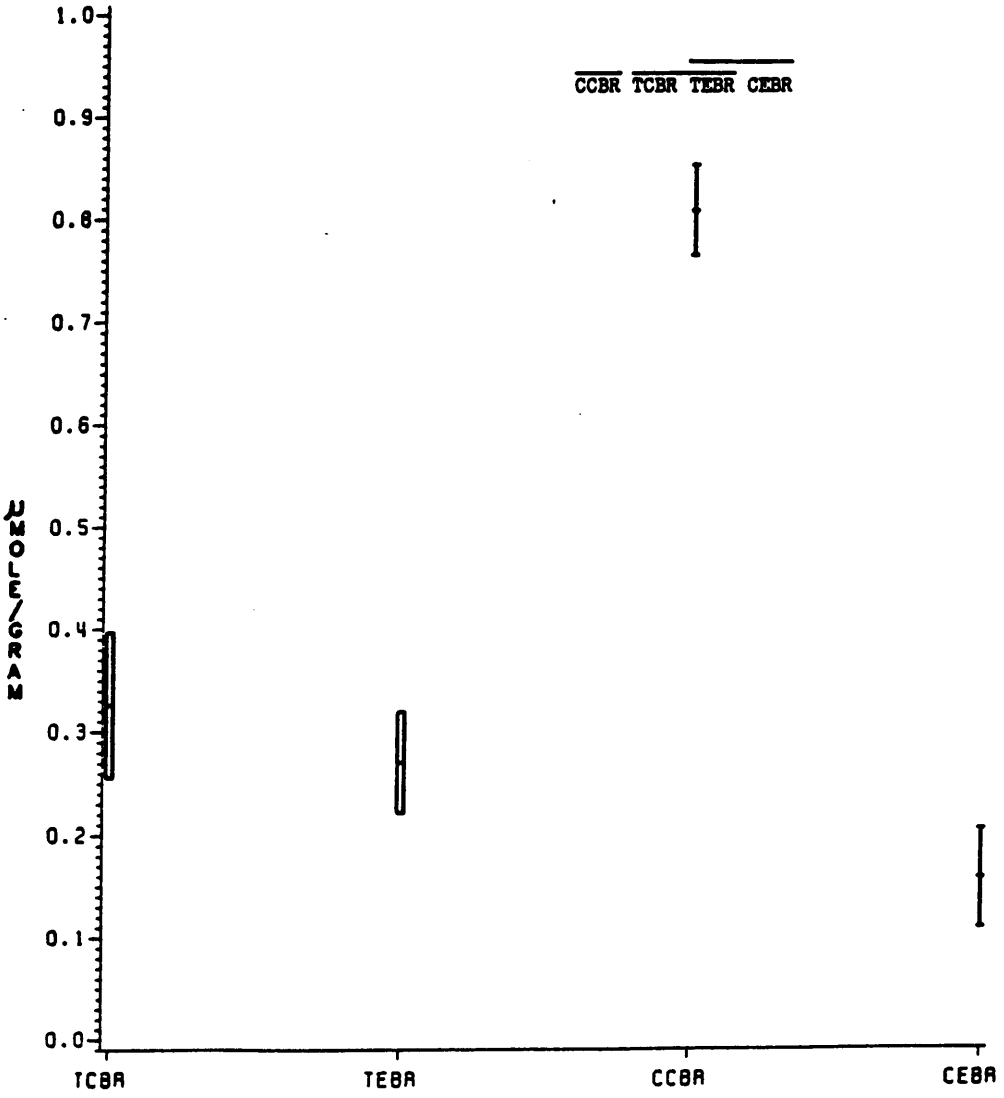

FIGURE 11. Creatine phosphate (Mean±1S.E.M.) TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; Groups under the same line are not significantly different at the p=0.05 level. N=9-11.

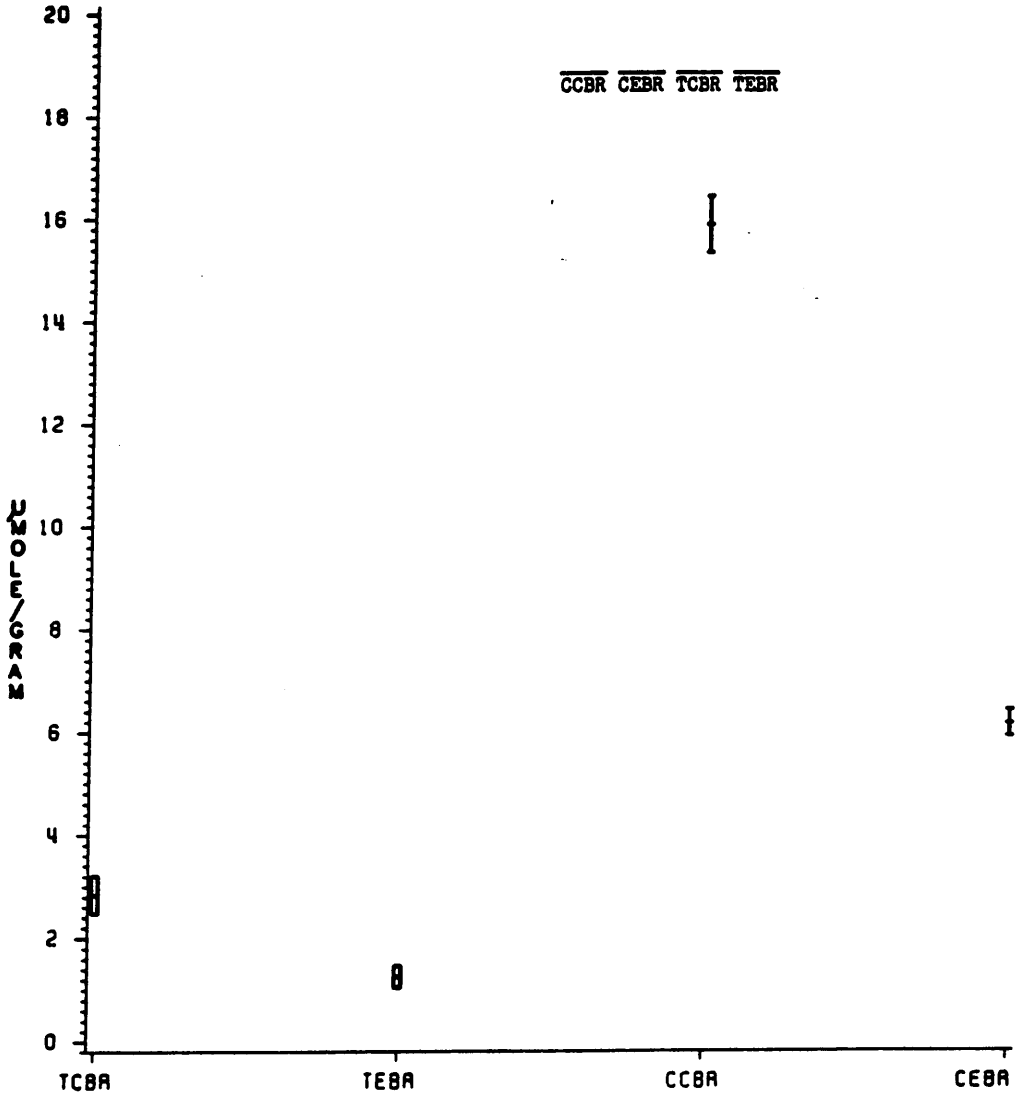

FIGURE 12. Glycogen (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain;
CCBR=Catfish control brain;CEBR=Catfish experimental brain;
Groups under the same line are not significantly different
at the p=0.05 level. N=10-11.

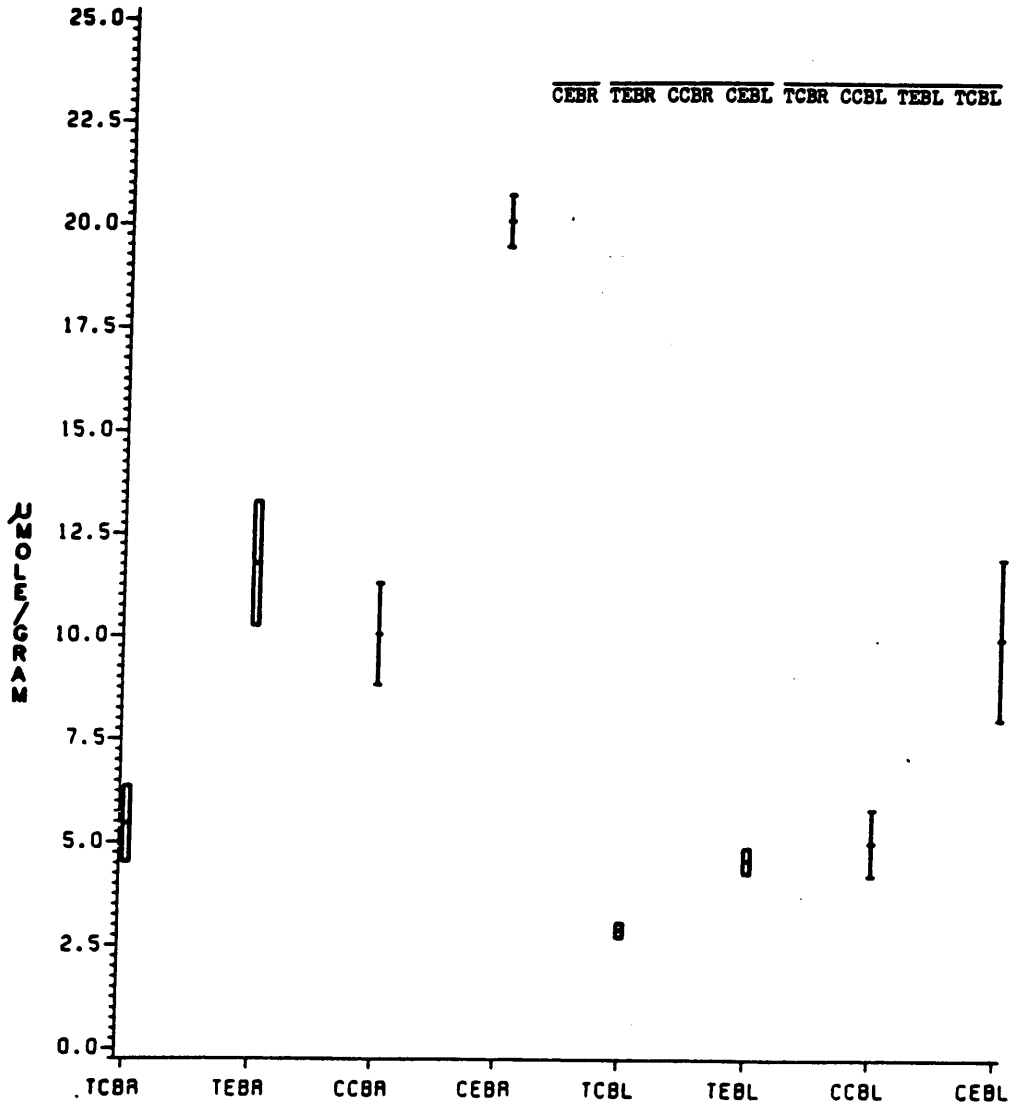

FIGURE 13. Lactate (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; TCBL=Trout control blood; TEBL=Trout experimental blood; CCBL=Catfish control blood;CEBL=Catfish experimental blood; Groups under the same line are not significantly different at the p=0.05 level. N=10-11 for brain and N=5 for blood.

There are a variety of endproducts which arise from alternative anaerobic pathways; this study examined six of those endproducts. The simultaneous catabolism of carbohydrates and amino acids has been implicated in the accumulation of several endproducts, succinate being one of them. With anoxia, trout brain and blood had a non-significant increase in succinate levels (Figure 14). Succinate in the brain of anoxic catfish tended to be lower than control values while anoxic blood was higher than the normoxic values. Brain levels for trout and catfish appeared higher than their respective blood levels. Alanine is a second suggested metabolite; however, neither trout nor catfish brains showed a significant increase in this compound following anoxia (Figure 15). Propionate concentrations were not significantly higher in the brains of either species following exposure to anoxia; levels in anoxic blood of both species tended to be lower than in their respective controls (Figure 16). Anoxic trout brain levels were higher than the blood values.

In trout and catfish, anoxic brain levels of isobutyrate (Figure 17) were not higher than control values. Blood of anoxic trout was significantly higher than brain and normoxic blood. In catfish, the concentration in normoxic blood was significantly lower than brain and anoxic blood values. The levels of isovalerate (Figure 18) did not change in trout brain or blood following exposure

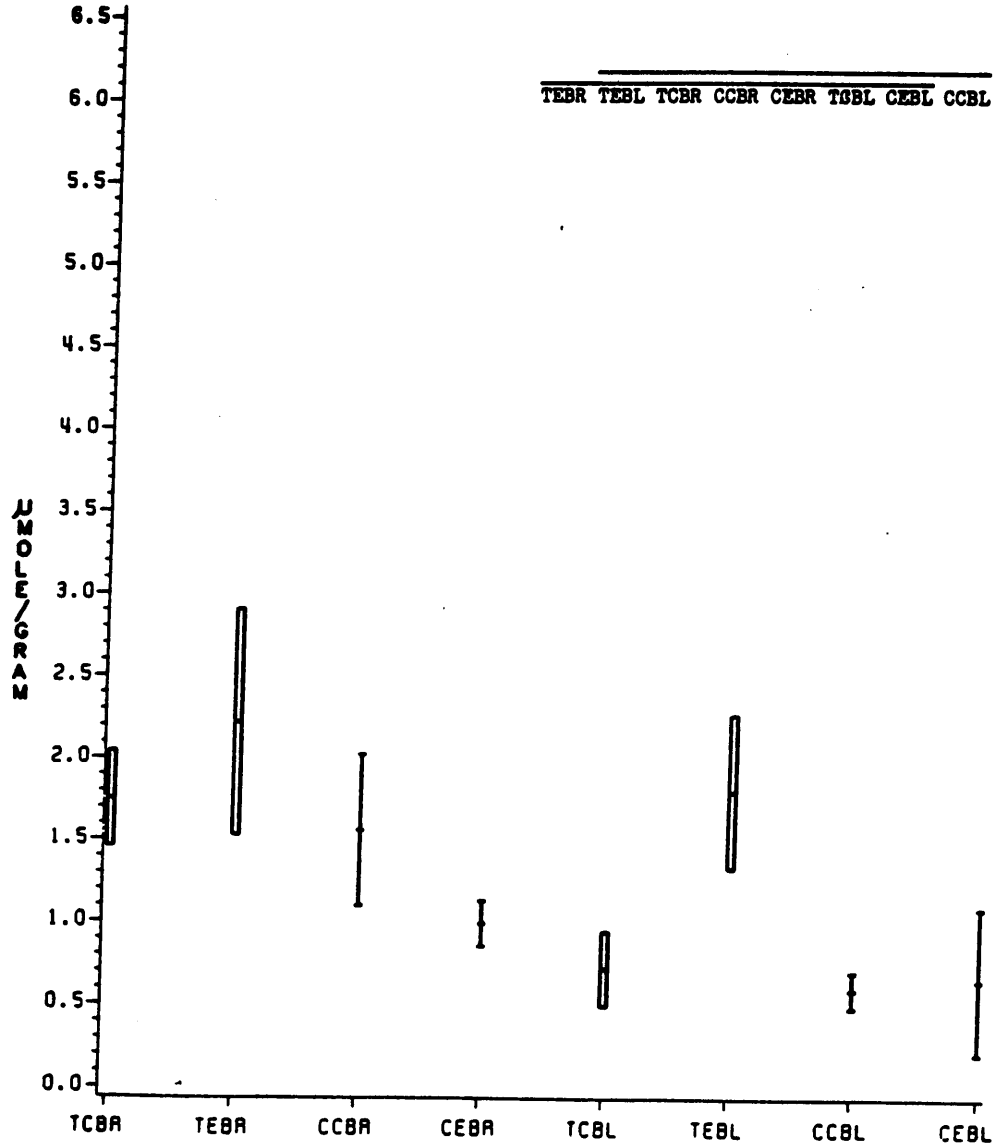

FIGURE 14. Succinate (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; TCBL=Trout control blood; TEBL=Trout experimental blood; CCBL=Catfish control blood;CEBL=Catfish experimental blood; Groups under the same line are not significantly different at the p=0.05 level. N=6-10 for brain and N=5 for blood.

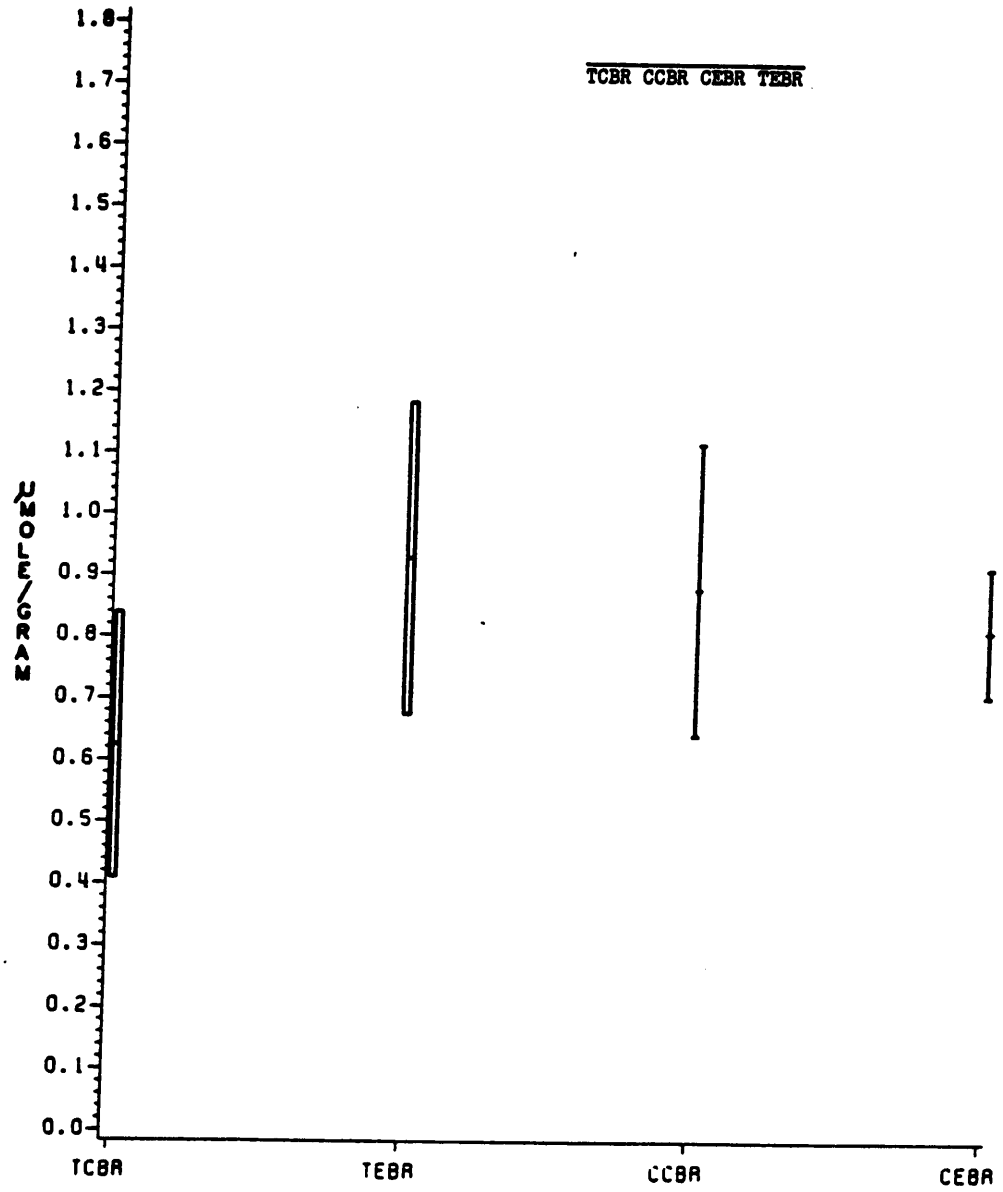

FIGURE 15. Alanine (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; Groups under the same line are not significantly different at the p=0.05 level. N=4-5.

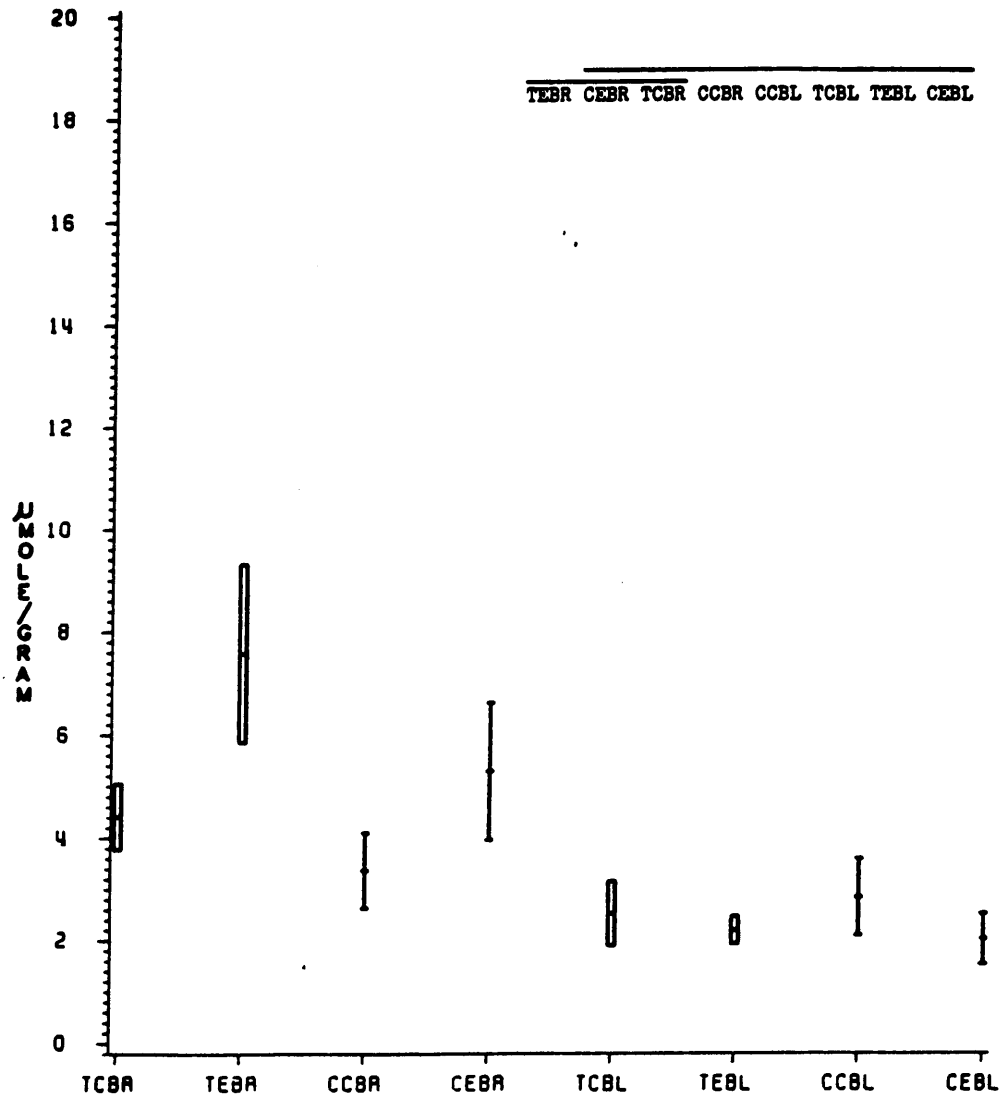

FIGURE 16. Propionate (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain;
CCBR=Catfish control brain;CEBR=Catfish experimental brain;
TCBL=Trout control blood; TEBL=Trout experimental blood;
CCBL=Catfish control blood;CEBL=Catfish experimental blood;
Groups under the same line are not significantly different
at the p=0.05 level. N=8-10 for brain and N=4-5 for blood.

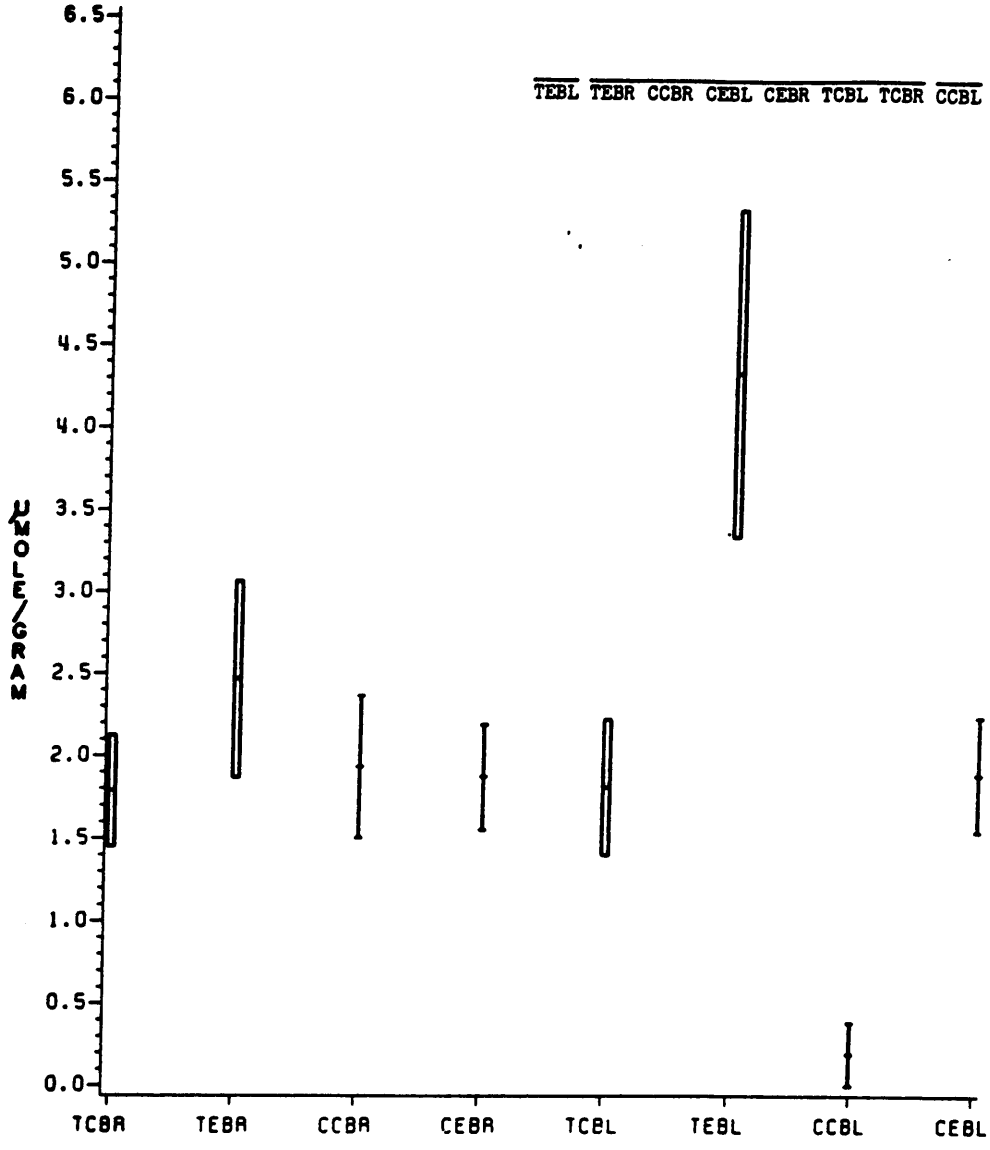

FIGURE 17. Isobutyrate (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; TCBL=Trout control blood; TEBL=Trout experimental blood; CCBL=Catfish control blood;CEBL=Catfish experimental blood; Groups under the same line are not significantly different at the p=0.05 level. N=7-10 for brain and N=5 for blood.

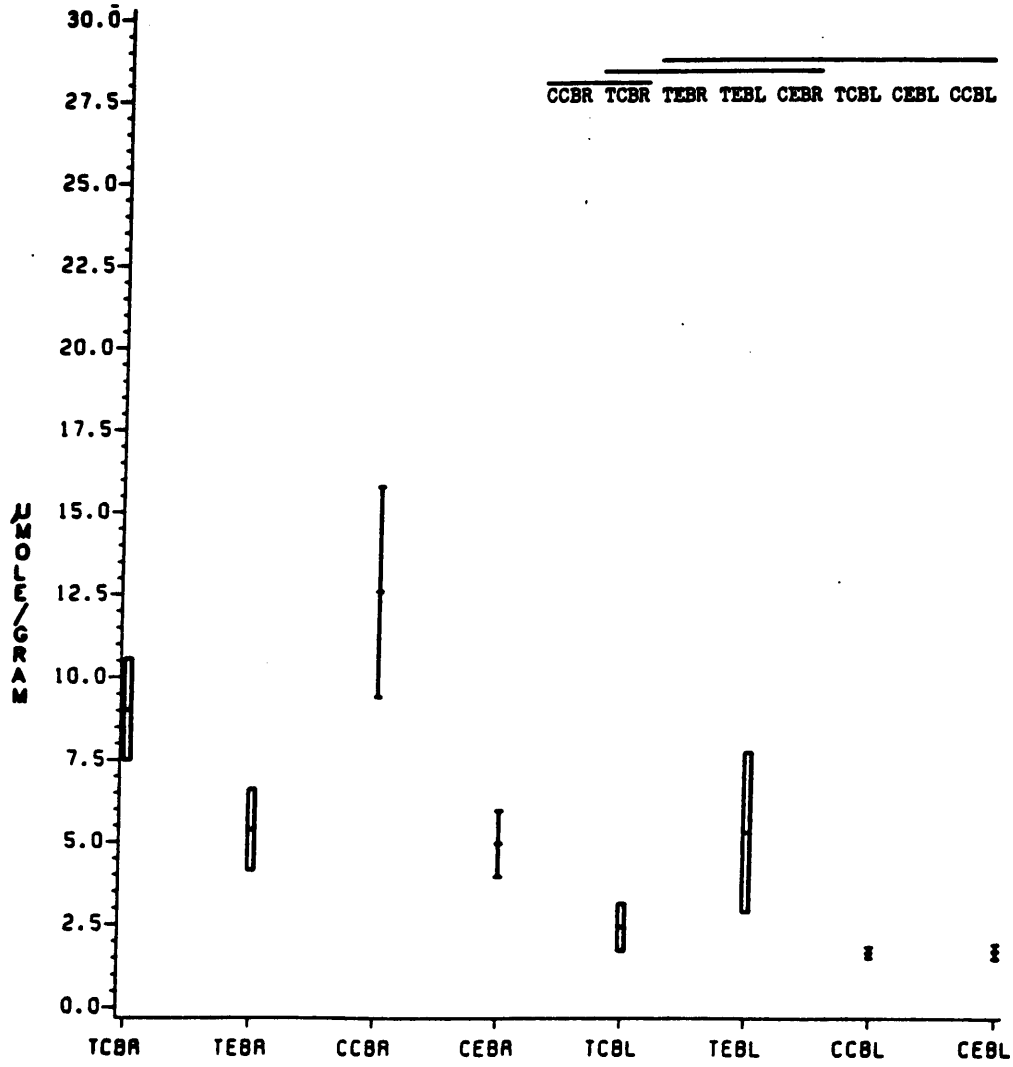

FIGURE 18. Isovalerate (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain;
CCBR=Catfish control brain;CEBR=Catfish experimental brain;
TCBL=Trout control blood; TEBL=Trout experimental blood;
CCBL=Catfish control blood;CEBL=Catfish experimental blood;
Groups under the same line are not significantly different
at the p=0.05 level. N=9-10 for brain and N=5 for blood.

to anoxia. Bullheads showed a significant decrease in brain levels with anoxia but blood levels were unchanged.

Ethanol is thought to accumulate from lactate, allowing redox balance and glycolysis to be maintained. There was no significant change in ethanol content of either trout or catfish brains following anoxia (Figure 19).

4.5 Analyses of Miscellaneous Compounds

Use of the HPLC also allowed the detection of three TCA cycle intermediates from the same sample. Alpha-ketoglutarate (Figure 20) did not accumulate in anoxic trout brain and blood; catfish also had no significant changes. Catfish brain levels were lower than trout brain levels. Pyruvate (Figure 21) and acetate (Figure 22) displayed no tissue nor species differences during anoxia. All values were within the range of previous studies (Smith and Heath 1980; Thillart et al. 1976).

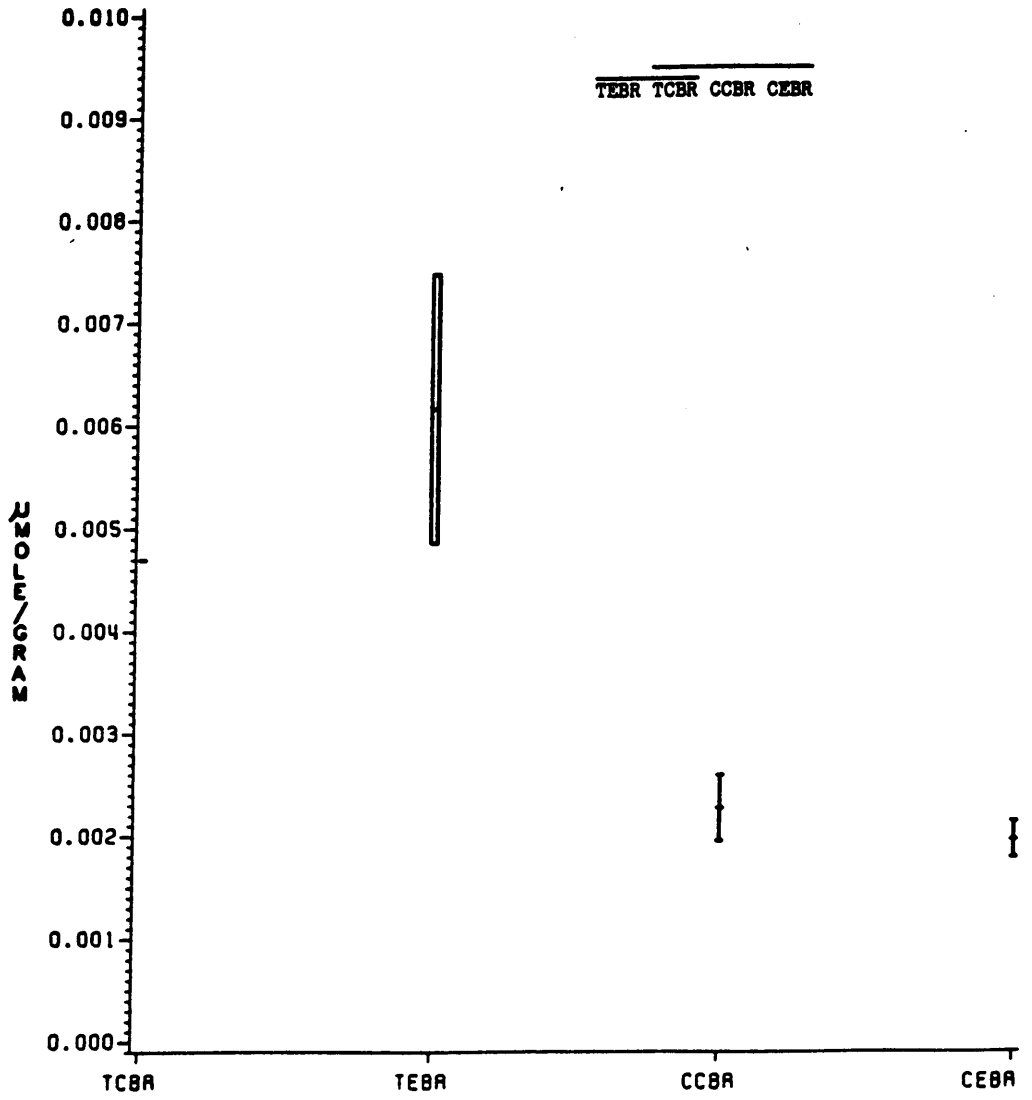

FIGURE 19. Ethanol (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; Groups under the same line are not significantly different at the p=0.05 level. N=3-4.

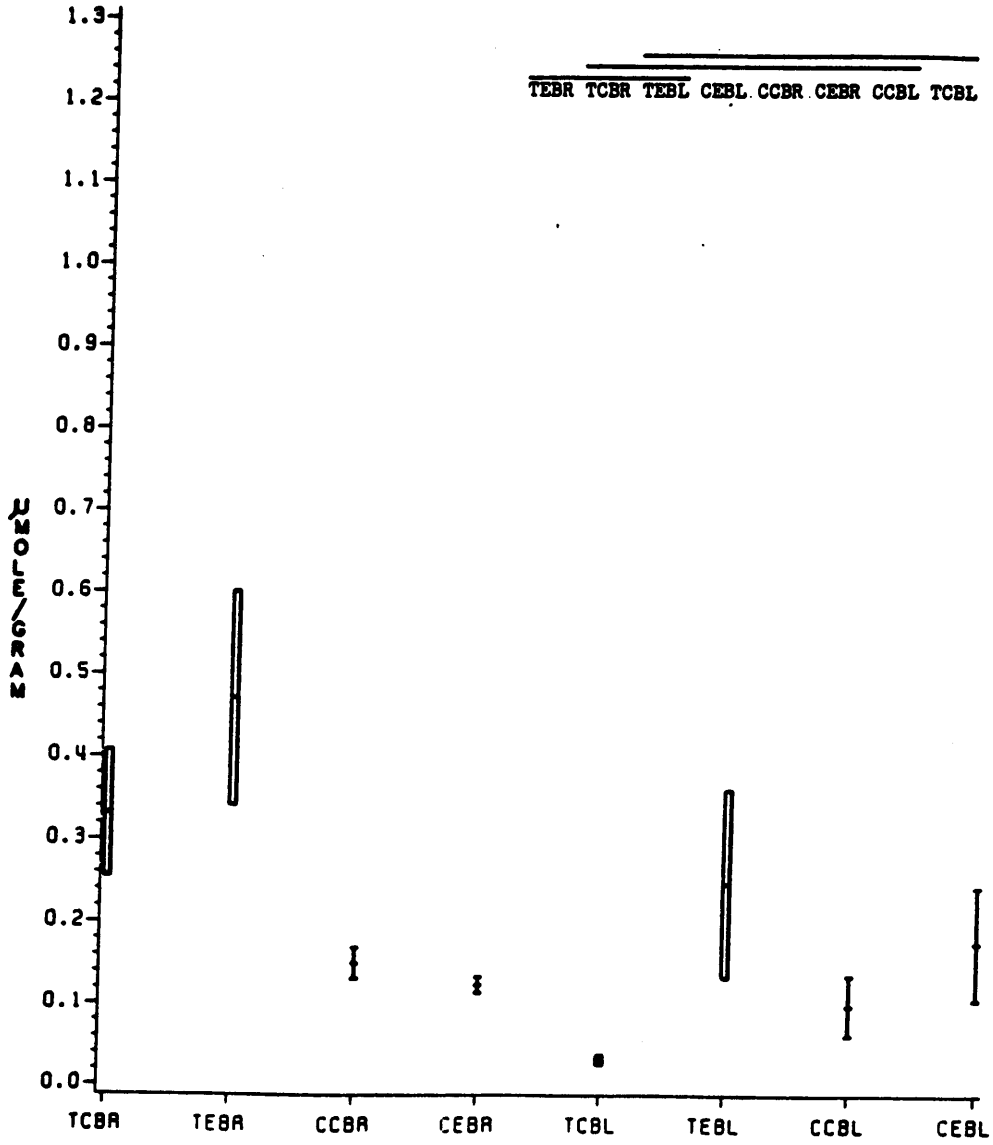

FIGURE 20. Alpha-ketoglutarate (Mean±1S.E.M.) TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; TCBL=Trout control blood; TEBL=Trout experimental blood; CCBL=Catfish control blood;CEBL=Catfish experimental blood; Groups under the same line are not significantly different at the p=0.05 level. N=9-10 for brain and N=5 for blood.

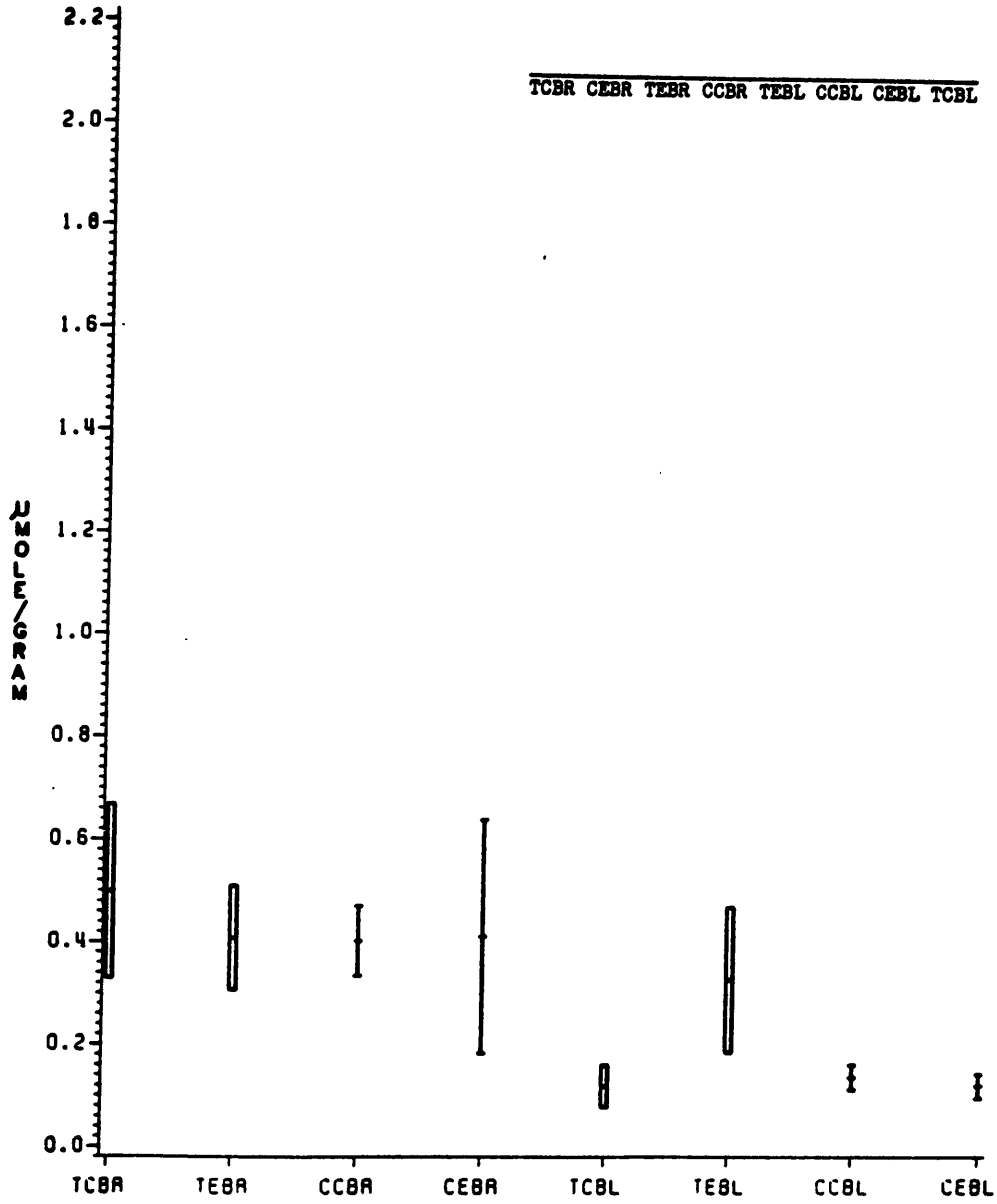

FIGURE 21. Pyruvate (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain; CCBR=Catfish control brain;CEBR=Catfish experimental brain; TCBL=Trout control blood; TEBL=Trout experimental blood; CCBL=Catfish control blood;CEBL=Catfish experimental blood; Groups under the same line are not significantly different at the p=0.05 level. N=9-11 for brain and N=5 for blood.

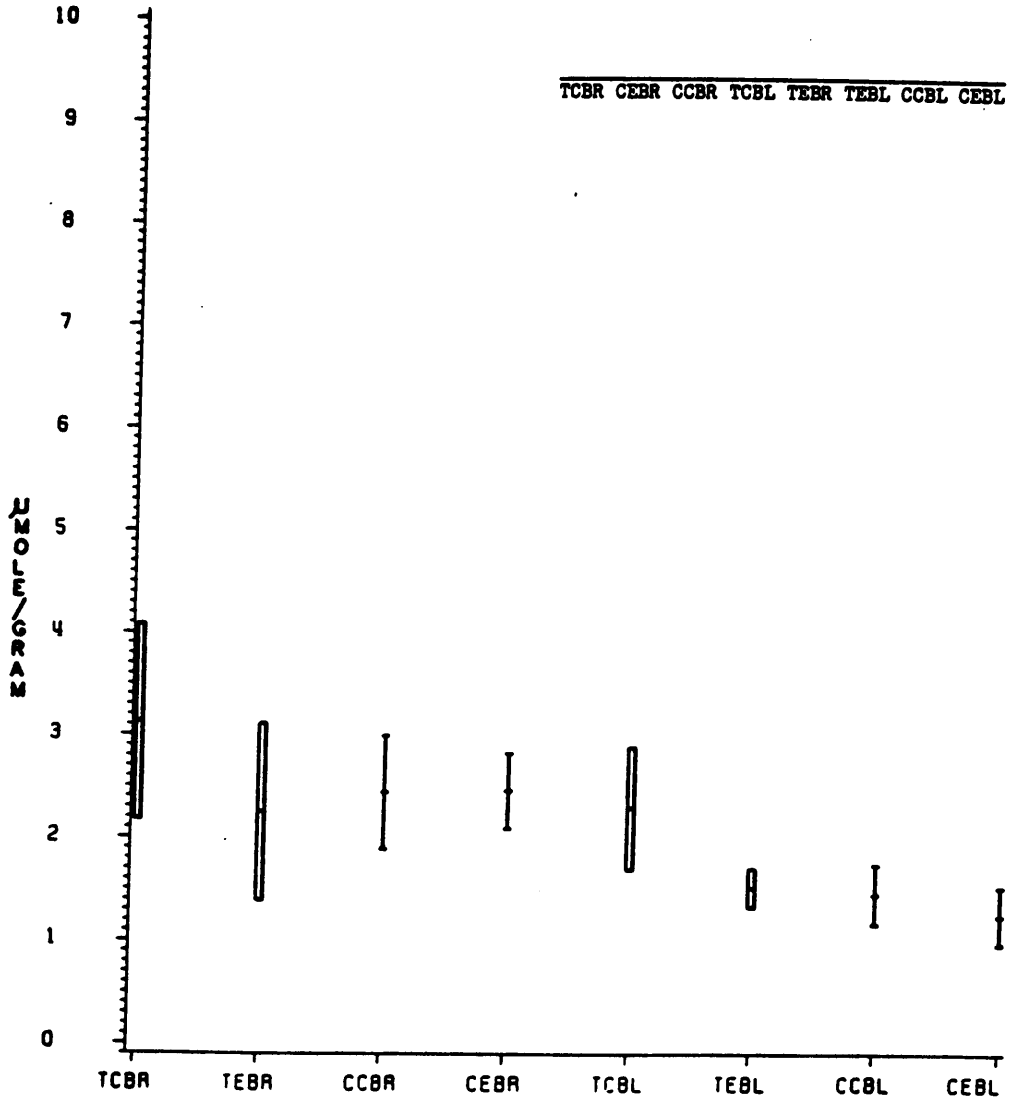

FIGURE 22. Acetate (Mean±1S.E.M.)
TCBR=Trout control brain; TEBR=Trout experimental brain;
CCBR=Catfish control brain;CEBR=Catfish experimental brain;
TCBL=Trout control blood; TEBL=Trout experimental blood;
CCBL=Catfish control blood;CEBL=Catfish experimental blood;
Groups under the same line are not significantly different
at the p=0.05 level. N=9-11 for brain and N=4-5 for blood.

5. DISCUSSION

The mechanisms employed in the brains of fish to survive anoxia have been studied very little. In mammals, the brain, compared to other organs, is the least capable of withstanding oxygen deprivation (Hochachka 1980; Liere and Stickney 1963); the same is probable for fish brains. Maintenance of energy within the fish brain is necessary for the continued survival of the animal because regulation of visceral functions and breathing is controlled by the brain (Marshall and Hughes 1980; Shelton 1970). In my study, the animals were considered brain dead when opercular movements stopped. Cessation of the heart was not used as an indicator of death because it was able to continue beating up to one hour after ventilation had ceased. The time to death in trout (12 minutes) corresponds to that of Smith and Heath (1980) for exposure to acute anoxia. No studies were found for brown bullheads but there are numerous anecdotal reports of this species surviving long periods out of the water, in part due to skin respiration.

Exposure to acute anoxia is admittedly an unnatural situation. Such treatment was chosen, however, to eliminate changes in anaerobic metabolism, hemoglobin affinity, and oxygen extraction efficiency as variables which can occur with a gradual lowering of oxygen tension (Smith and Heath

1980; Thillart 1982). Both species probably decreased their energy use, both in the brain and rest of the body during the period of anoxia (Hochachka 1985; Sick et al. 1984; Thillart 1982). Since the trout and bullhead exhibited similar behavior in the chamber, it is, however, unlikely that the ability of bullhead to survive anoxia six times longer than trout is due primarily to the bullhead reducing its energy use in the brain to a level significantly lower than in the trout brain. Systemic oxygen reserves should not account for these differences since such reserves would permit the generation of less than one-third of the necessary ATP in a resting fish (Thillart et al. 1976). Turtles, which are quite resistant to anoxia, do not use these reserves to maintain energy in the brain (Sick et al. 1984).

No values for ATP concentrations in normoxic trout or bullhead brains were found in the literature. The level obtained for trout (0.75um/g) is less than the few reported values for other species. Bullheads, however, had initial levels (1.62um/g) similar to goldfish, frog, and turtle brain values reported by McDougal et al. (1968) but less than normoxic values (1.9 to 13um/g) reported by Shaffi (1980). Bullhead levels were reduced at death to 0.96um/g. This is higher than the value of 0.2um/g reported by McDougal et al. (1968) for goldfish, frog, and turtle brains at death from anoxia. It is surprising to note that

trout did not exhibit a significant decrease in ATP levels (0.68um/g) at death following anoxia.

The catabolism of free glucose generates ATP, but in this study, neither trout nor bullhead exhibited decreased levels of glucose. Studies involving muscle have found no change or increased levels during hypoxia or anoxia (Johnston 1975a,b; Johnston and Bernard 1983; Jorgensen and Mustafa 1980a). McDougal et al. (1968) noted a decrease in brain glucose in goldfish and frog (5.3 to 1.0um/g and 2.0 to 0.4um/g respectively). In that study, anoxia was produced by decapitation so any supply of glucose from the blood was eliminated. Values for catfish (3.7um/g) are close to those found by McDougal et al.(1968), while trout (0.8um/g) are near initial levels reported for turtle brains (0.5um/g). Supplies of glucose probably are not the limiting factor for either the trout or the bullhead.

In mammals, ketone bodies transfer acetyl groups, derived from lipids, from the liver to other tissues, including the brain. Beta-hydroxybutyrate is converted to acetoacetate which is then converted to its CoA derivative for use in the TCA cycle (Lehninger 1982). There is little information regarding these compounds and their role in fish, but it is assumed that the brain can use these compounds. The values obtained in this study are considerably higher than those reported by Thillart et al.

(1982); perhaps this is due to the increased resolution and sensitivity of the HPLC. The trout brain showed increased levels of B-hydroxybutyrate while blood did not change with anoxia. Thillart et al. (1982) reported increased levels in muscle and liver of trout following anoxia, with no change in blood. Bullheads showed no change in brain or blood but both species tended to have higher levels in the brain than in the blood. Acetoacetate in this study, as in that of Thillart et al. (1982), did not change with exposure to anoxia; brain levels were higher than blood levels. Hochachka (1980) has suggested that B-hydroxybutyrate may accumulate during anoxia because B-hydroxybutyrate dehydrogenase helps regulate NADH (Acetoacetate + NADH + H^+ <====> B-hydroxybutyrate + NAD^+). Beta-hydroxybutyrate levels were higher than acetoacetate levels suggesting that either more B-hydroxybutyrate is produced than acetoacetate or the above reaction is used to maintain redox balance. My work also suggests that ketone bodies are a source of energy to fish brains during normoxia.

Creatine phosphate is a storage form of high-energy phosphate groups. Decreased concentrations of this compound have been noted in muscle and liver of goldfish (Thillart et al. 1980) and carp (Johnston and Bernard 1983) following anoxia while plaice showed no change (Jorgensen and Mustafa 1980b). Levels of creatine phosphate in these studies ranged from 0.2um/g in liver to 8.7um/g in white muscle.

McDougal et al. (1968) found goldfish brain stores decreased from 3.9 to 0.2um/g with anoxia; a similar decrease was noted in frog and turtle brains. Initial values for trout and bullhead (0.33 and 0.81um/g) in my study were much less than the values of McDougal et al. (1968). Levels in trout did not decrease significantly, suggesting that the brain can generate enough ATP through anaerobic pathways. Levels of creatine phosphate in the bullhead brain, however, decreased over 80% to 0.16um/g. Goldfish brains had their levels reduced by 95% during anoxia (McDougal et al. 1968). The brains of bullheads, as with the goldfish, appear to use their CrP reserves to help maintain ATP levels.

The main storage form of glucose is glycogen, which consists of many glucose residues. Values for glycogen have been reported for many species and tissues, with the general trend being a reduction in stores with exposure to anoxia (Johnston 1975b; Thillart et al 1976, 1980). Levels in the brains of goldfish, frog, and turtle averaged 16um/g and decreased to 5um/g following anoxia (McDougal et al. 1968). In my study, trout brain initially contained 2.8um/g and catfish 15.8um/g. Each exhibited a greater than 50% reduction in glycogen with anoxic exposure. It appears that each species is utilizing its glycogen stores to generate energy anaerobically. The detection of glycolytic enzymes

has been reported in the brain of fish. The capacity of the brain to carry out glycolysis is second only to skeletal muscle and similar to heart muscle (Hochachka 1970; Knox et al. 1980). It is interesting to note that catfish brains contain close to six times more glycogen than trout and are also able to survive anoxia approximately six times longer.

Lactate is the endproduct of classical anaerobic glycolysis. Numerous studies indicate an increase in muscle and liver lactate following anoxia (Burton and Heath 1980; Driedzic and Hochachka 1975; Johnston 1975a,b; Smith and Heath 1980). It appears that the brains of trout and bullheads are able to generate ATP through the use of anaerobic glycolysis. After exposure to anoxia, there was an approximately 100% increase in anoxic brain and blood lactate levels when compared to their respective controls. The concentration of lactate in the brain was always above that of the blood so the source of lactate in the brain was endogenous. Goldfish and frog brains exhibited a much greater increase with levels increasing from 4 to 34um/g (McDougal et al. 1968), perhaps due to their inability to eliminate lactate from the brain via the blood because anoxia was produced by decapitation.

Although brain lactate levels were almost double the blood levels, there is little evidence to suggest gluconeogenesis from lactate by the brain. Conversions of

lactate into glycogen and ethanol in muscle have been proposed (Batty and Wardle 1979; Shoubridge and Hochachka 1980); the enzymes involved have not been found in the brains of fish (Knox et al. 1980; Shoubridge and Hochachka 1980). It is, therefore, unlikely that lactate is taken up by the brain from the blood but rather that lactate is produced by the brain, perhaps in amounts toxic to the animal (Belkin 1968).

The accumulation of alternative anaerobic endproducts has been documented in molluscs, helminths, and leeches. Succinate is one suggested alternative endproduct resulting from the simultaneous anaerobic catabolism of glucose and amino acids. Slight increases in muscle succinate of anoxic rainbow trout have been reported (Johnston 1975b; Smith and Heath 1980), but neither trout nor bullheads exhibited a significant increase in brain or blood succinate. Alanine may also accumulate from this pathway, but again, neither trout nor catfish brain exhibited any accumulation of alanine. All values for succinate and alanine were within previously reported ranges. Propionate is thought to accumulate as an anaerobic endproduct from succinate in some invertebrates. This study does not show a significant increase in propionate levels of brain or blood in trout or catfish. Studies on other tissues of fish have also failed to find increases of this endproduct after anoxia (Driedzic

and Hochachka 1975; Thillart et al. 1976).

Isobutyrate and isovalerate have been found to accumulate in helminths during anoxia; no significant accumulation of these compounds in anoxic fish has not been reported (Driedzic and Hochachka 1975). Anoxic trout blood had significantly higher levels of isobutyrate than other brain or blood values. This may indicate production and subsequent release to the blood by some other organ. Isovalerate levels decreased following anoxia exposure; the significance of this is hard to estimate since the number of studies concerning this compound in fish tissues is limited. Levels are higher than those reported by Driedzic and Hochachka (1975) and may be attributable to differences in means of detection of these compounds. In general, it appears that the brains of trout and catfish derive essentially no energy through the use of the alternative anaerobic pathways described in known facultative anaerobes.

Ethanol has been reported to accumulate in tissue and surrounding water of anoxic goldfish and Crucian carp (Johnston and Bernard 1983; Shoubridge and Hochachka 1980). Lactate is the suggested substrate of ethanol formation. Increases in ethanol concentration were not detected in trout or bullhead brains,nor in their water. Unlike the goldfish, it does not appear that these species transport lactate to the muscles for conversion to ethanol

as suggested by Shoubridge and Hochachka (1980).

It appears that the brain of bullhead catfish is able to maintain an energy supply using anaerobic glycolysis as its primary metabolic pathway. The brain of this species stores energy in the form of creatine phosphate and glycogen, depleting them during anoxia. At some point however, the brain will not be able to maintain a minimal, but necessary, ATP level. This will most likely result in the loss of the membrane ion gradients which are so vital to the transmission of nerve impulses (McDougal et al. 1968; Sick et al. 1984).

The death of the trout is not as easily explained by these data. From this study, it appears that the trout brain had enough energy in the forms of ATP, CrP, and glucose to maintain the integrity of the brain. Hochachka (1985) has suggested that animals which are tolerant of anoxia have cell membranes (especially in nervous tissue) which are less permeable to electrolytes; maintenance of the proper ion gradients may occur even if ATP levels decrease. Perhaps the trout cannot maintain its ionic gradients because its membranes are more permeable than those of the bullhead, resulting in a loss of impulse transmission. Death might also result from injury due to the build up of lactic acid within the brain. Hochachka (1985) has proposed that, during anoxia, a large influx of

calcium into a cell causes destruction of the cell membranes through activation of phospholipases. It may be that the lactic acid causes the release of intracellular calcium stores. The increased calcium levels activate the phospholipases and the cell membrane is destroyed. Nerve transmission cannot occur because of the leakiness of the membrane and death results.

6. SUMMARY

Bullhead catfish survived total anoxia for a period of time six-fold longer than could rainbow trout. The results of this study show that the brain of bullhead catfish stores large amounts of glycogen. The brain is able to utilize these stores via classical anaerobic glycolysis upon exposure to anoxia. Bullhead brain tissue does not appear to possess any alternative anaerobic pathways as are found in facultative anaerobes. The stores of creatine phosphate are depleted to maintain ATP levels during anoxia. Death is most likely due to an inability to maintain ATP levels which could lead to the loss of ion gradients so vital to the propagation of nerve impulses.

The rainbow trout does not appear to store large amounts of glycogen in the brain and these become depleted during anoxia. Creatine phosphate reserves are not reduced with exposure to anoxia nor does the brain of the trout experience a significant decrease in ATP levels. Death does not appear to be due to the loss of energy from the brain. Perhaps death results from lactic acid build up which leads to the activation of phospholipases. These enzymes destroy the cell membrane, causing an inability to transmit nerve impulses.

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8. APPENDIX

TABLE 4. Summary of results pertaining to control trout

	Mean	±S.E.M.	Range	N
Brain				
Weight[1]	0.314	0.018	0.236-0.463	12
TTD[2]	12.67	1.499	9.00-27.000	12
ATP[3]	0.752	0.101	0.235-1.248	12
CrP	0.326	0.070	0.000-0.655	11
Glc	0.921	0.071	0.621-1.270	10
Gly[4]	2.844	0.365	0.494-4.467	11
EtOH	0.005	0.000	0.005-0.005	3
Ala	0.624	0.215	0.064-1.053	5
Keto	0.331	0.076	0.069-0.724	10
Pyr	0.499	0.169	0.038-1.486	10
Succ	1.750	0.290	0.240-3.244	10
Lact	5.443	0.917	2.51-11.705	11
Beta	7.769	0.722	4.31-11.720	10
Aceto	1.888	0.435	0.708-4.105	9
Acetat	3.126	0.952	0.929-9.012	11
Prop	4.413	0.644	2.526-7.095	9
But	1.787	0.336	0.664-3.909	10
Val	9.030	1.525	1.635-16.54	10
Blood				
Weight	0.200	0.000	0.200-0.200	5
TTD	9.800	0.200	9.000-10.00	5
Keto	0.031	0.006	0.008-0.041	5
Pyr	0.118	0.041	0.049-0.270	5
Succ	0.722	0.225	0.381-1.609	5
Lact	2.894	0.177	2.384-3.334	5
Beta	3.863	0.567	2.481-5.524	5
Aceto	1.548	0.607	0.476-3.899	5
Acetat	2.283	0.596	1.427-4.623	5
Prop	2.520	0.631	1.212-4.554	5
But	1.812	0.412	0.640-2.748	5
Val	2.455	0.716	1.273-5.124	5

[1]Tissue wet weight in grams
[2]Time to death in minutes
[3]Remaining values expressed as micromoles per gram tissue wet weight
[4]Glycogen expressed as micromoles glucosyl units per gram tissue wet weight

TABLE 5. Summary of results pertaining to anoxic trout

	Mean	±S.E.M.	Range	N
Brain				
Weight[1]	0.338	0.020	0.219-0.435	11
TTD[2]	12.00	1.342	7.00-21.000	11
ATP[3]	0.681	0.096	0.262-1.253	11
CrP	0.270	0.049	0.000-0.502	11
Glc	0.676	0.064	0.419-1.002	11
Gly[4]	1.254	0.209	0.000-2.387	11
EtOH	0.006	0.001	0.004-0.009	4
Ala	0.927	0.253	0.277-1.804	5
Keto	0.470	0.129	0.080-1.212	10
Pyr	0.408	0.101	0.030-1.233	11
Succ	2.217	0.686	0.580-6.255	10
Lact	11.76	1.509	3.02-18.996	11
Beta	14.31	1.841	5.32-24.166	11
Aceto	5.194	1.624	1.086-16.86	9
Acetat	2.248	0.861	0.546-9.017	10
Prop	7.578	1.725	2.643-19.76	10
But	2.466	0.599	0.362-5.423	11
Val	5.417	1.235	1.122-12.42	10
Blood				
Weight	0.200	0.000	0.200-0.200	5
TTD	11.40	1.913	7.000-16.00	5
Keto	0.245	0.114	0.016-0.643	5
Pyr	0.324	0.141	0.125-0.857	5
Succ	1.801	0.469	0.999-3.604	5
Lact	4.577	0.308	3.913-5.616	5
Beta	5.541	1.299	2.904-9.980	5
Aceto	2.827	1.407	0.110-6.780	5
Acetat	1.513	0.188	1.297-2.074	4
Prop	2.186	0.262	1.619-2.881	4
But	4.328	0.994	1.442-6.334	5
Val	5.338	2.412	1.174-14.44	5

[1]Tissue wet weight in grams
[2]Time to death in minutes
[3]Remaining values expressed as micromoles per gram tissue wet weight
[4]Glycogen expressed as micromoles glucosyl units per gram tissue wet weight

TABLE 6. Summary of results pertaining to control catfish

	Mean	±S.E.M.	Range	N
Brain				
Weight[1]	0.279	0.008	0.240-0.309	10
TTD[2]	72.70	8.86	40.0-120.00	10
ATP[3]	1.621	0.104	1.128-2.099	9
CrP	0.806	0.044	0.578-0.976	9
Glc	3.699	0.256	2.268-4.669	10
Gly[4]	15.85	0.543	12.76-19.38	10
EtOH	0.002	0.000	0.002-0.003	3
Ala	0.877	0.237	0.210-1.248	4
Keto	0.148	0.019	0.087-0.277	10
Pyr	0.401	0.068	0.086-0.709	9
Succ	1.559	0.461	0.272-3.045	6
Lact	10.04	1.236	3.31-15.185	10
Beta	10.80	1.053	5.44-14.466	9
Aceto	3.986	0.589	1.150-6.006	9
Acetat	2.440	0.554	0.984-5.647	9
Prop	3.361	0.739	1.299-7.359	8
But	1.932	0.429	0.500-4.002	7
Val	12.61	3.192	4.015-27.94	9
Blood				
Weight	0.200	0.000	0.200-0.200	5
TTD	76.00	14.09	40.0-120.00	5
Keto	0.097	0.036	0.032-0.239	5
Pyr	0.135	0.023	0.080-0.206	5
Succ	0.596	0.109	0.243-0.919	5
Lact	5.000	0.804	2.444-7.049	5
Beta	8.829	1.229	6.21-11.784	5
Aceto	0.989	0.063	0.872-1.086	3
Acetat	1.448	0.287	0.763-2.207	5
Prop	2.825	0.745	0.913-4.518	5
But	0.191	0.191	0.000-0.956	5
Val	1.690	0.169	1.308-2.216	5

[1]Tissue wet weight in grams
[2]Time to death in minutes
[3]Remaining values expressed as micromoles per gram tissue wet weight
[4]Glycogen expressed as micromoles glucosyl units per gram tissue wet weight

TABLE 7. Summary of results pertaining to anoxic catfish

	Mean	±S.E.M.	Range	N
Brain				
Weight[1]	0.334	0.013	0.269-0.398	10
TTD[2]	62.20	5.625	41.00-91.00	10
ATP[3]	0.959	0.143	0.168-1.482	10
CrP	0.155	0.048	0.000-0.406	10
Glc	3.900	0.315	2.120-5.441	10
Gly[4]	6.150	0.261	4.691-7.683	10
EtOH	0.002	0.000	0.001-0.002	4
Ala	0.808	0.104	0.492-1.108	5
Keto	0.122	0.009	0.063-0.170	9
Pyr	0.408	0.226	0.043-2.122	9
Succ	0.991	0.138	0.416-1.623	8
Lact	20.08	0.635	17.30-24.16	10
Beta	9.387	0.888	6.15-14.179	9
Aceto	5.498	0.614	2.240-8.602	10
Acetat	2.453	0.365	1.287-4.766	9
Prop	5.281	1.330	1.378-12.97	9
But	1.873	0.319	0.454-3.079	9
Val	4.985	1.001	2.303-11.25	9
Blood				
Weight	0.200	0.000	0.200-0.200	5
TTD	65.00	7.043	41.00-85.00	5
Keto	0.173	0.069	0.042-0.424	5
Pyr	0.118	0.023	0.050-0.169	5
Succ	0.652	0.439	0.000-2.204	5
Lact	9.933	1.949	2.18-12.429	5
Beta	7.262	0.532	5.665-8.294	5
Aceto	2.949	1.512	0.260-6.571	4
Acetat	1.241	0.277	0.574-1.866	5
Prop	2.007	0.493	0.997-3.518	5
But	1.888	0.348	1.022-2.908	5
Val	1.741	0.224	1.315-2.297	5

[1]Tissue wet weight in grams
[2]Time to death in minutes
[3]Remaining values expressed as micromoles per gram tissue wet weight
[4]Glycogen expressed as micromoles glucosyl units per gram tissue wet weight

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