Cell volume changes affect gluconeogenesis in the perfused liver of the catfish *Clarias batrachus*

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In addition to lactate and pyruvate, some amino acids were found to serve as potential gluconeogenic substrates in the perfused liver of *Clarias batrachus*. Glutamate was found to be the most effective substrate, followed by lactate, pyruvate, serine, ornithine, proline, glutamine, glycine, and aspartate. Four gluconeogenic enzymes, namely phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase (PC), fructose 1,6-bisphosphatase (FBPase) and glucose 6-phosphatase (G6Pase) could be detected mainly in liver and kidney, suggesting that the latter are the two major organs responsible for gluconeogenic activity in this fish. Hypo-osmotically induced cell swelling caused a significant decrease of gluconeogenic efflux accompanied with significant decrease of activities of PEPCK, FBPase and G6Pase enzymes in the perfused liver. Opposing effects were seen in response to hyper-osmotically induced cell shrinkage. These changes were partly blocked in the presence of cycloheximide, suggesting that the aniso-osmotic regulations of gluconeogenesis possibly occurs through an inverse regulation of enzyme proteins and/or a regulatory protein synthesis in this catfish. In conclusion, gluconeogenesis appears to play a vital role in *C. batrachus* in maintaining glucose homeostasis, which is influenced by cell volume changes possibly for proper energy supply under osmotic stress.

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1. Introduction

Gluconeogenesis comprises the synthesis of glucose from substrates such as pyruvate, lactate, glycerol, Krebs cycle intermediates and certain amino acids. The high dietary requirements of protein in fishes provide adequate substrates for this pathway for finally utilizing glucose as an energy source together with the generally lowered energy demands of fish (Pandian and Vivekananda 1985). Further, carbohydrate may also be used for short-term responses in acute stress situations as a last resort in fish (Christiansen and Klungsøyr 1987). Gluconeogenesis has been extensively studied in the liver and kidney in various fish species, since both liver and kidney are the major sites of this metabolic pathway (Mommsen 1986; Moon 1988; Saurez and Mommsen 1987). In some teleostean fishes gluconeogenesis occurs at relatively higher rates (Cowey 1979; Hayashi and Ooshiro 1979; Renaud and Moon 1980a,b; Bever *et al* 1981; Moon *et al* 1985) and is thought to be a key process in maintaining glucose homeostasis (Carneiro and Amaral 1982), especially in carnivorous fishes that have

Keywords. Anisotonicity; cell volume; *Clarias batrachus*; fructose 1,6-bisphosphatase; gluconeogenesis; glucose 6-phosphatase; hydration status; osmotic stress; phosphoenolpyruvate carboxykinase; pyruvate carboxylase

Abbreviations used: FBPase, Fructose 1,6-bisphosphatase; GDH, glutamate dehydrogenase; GPase, glycogen phosphorylase; G6Pase, glucose-6-phosphatase; G6PDH, glucose-6-phosphate dehydrogenase; GSase, glycogen synthase; LDH, lactate dehydrogenase; PC, pyruvate carboxylase; PEPCK, phosphoenol-pyruvate carboxykinase; RVD, regulatory volume decrease; RVI, regulatory volume increase.

high protein and low carbohydrate diets (De la Higuera and Cardenas 1986).

A remarkable property of living cells is their ability to maintain a comparatively constant cell volume under different physiological conditions (Bianchini *et al* 1988; Michel *et al* 1994; Jackson *et al* 1996; Häussinger 1996; Perlman and Goldstein 1999). Cell volume may be challenged by a variety of factors which distort the osmotic equilibrium between the intra- and extra-cellular spaces primarily during intestinal absorption of water, and of various amino acids and metabolites; or by exposure to different osmotic environments especially in the case of aquatic animals. Although most of the cells possess various volume-regulatory mechanisms to maintain the constancy of cell volume and also the hydration status of the cell via the regulatory volume decrease (RVD) and regulatory volume increase (RVI) mechanisms largely by changing the permeability of various ions such as K^+, Na^+, H^+, Cl^- and HCO_3^-, it has been noticed in many cell types that they remain either in a slightly swollen or shrunken state for the duration of the aniso-osmotic exposure (for review, see Häussinger 1996). Irrespective of the route of RVD or RVI, increases in hepatic cell volume generally result in increased anabolism and curtailment of catabolic pathways, while the reverse is true during the decrease in hepatic cell volume (Saha *et al* 1992; Lang *et al* 2000; Weiergräber and Häussinger 2000).

Teleost fishes face problems of osmotic stress primarily due to osmolarity changes in their external environment, and also due to intestinal absorption of amino acids and other metabolites. The mechanism of cell volume regulation in fishes has been studied in detail (Bianchini *et al* 1988; Perlman and Goldstein 1999; Haynes and Goldstein 1993; Michel *et al* 1994; Jackson *et al* 1996), reports related to the alterations of different metabolic processes in relation to cell volume changes are not many. Recently, the effects of cell volume changes have been reported in the perfused liver of air-breathing catfish (*C. batrachus*) on glucose, pyruvate and lactate efflux along with changes of glycogen phosphorylase (GPase) and glycogen synthase (GSase) enzyme activity (Goswami and Saha 1998), and also on hexose monophosphate pathway (HMP) (Saha and Goswami 2004). Hallgren *et al* (2003) reported similar effects of cell volume changes on GPase activity and glucose production in the hepatocytes of three fish species and showed a direct correlation of GPase a (active) activity with the hydration status of the cell. In continuation of our investigations on the effects of cell volume changes on carbohydrate metabolism on air-breathing catfishes, we report here the tissue distribution of different gluconeogenic enzymes, the normal gluconeogenic efflux by the perfused liver of *C. batrachus* using different gluconeogenic substrates, and the effects of changes of hepatic cell volume or hydration status on gluconeogenesis.

2. Materials and methods

2.1 *Animals*

C. batrachus, weighing 100 ± 15 g body mass, were purchased from commercial sources and acclimatized in the laboratory approximately for 1 month at a constant room temperature (28 ± 2°C) with 12 h : 12 h light and dark photoperiod. Only male fishes were used while performing these studies. Minced pork liver and rice bran (5% of the body mass) was given as food, and the water (collected from a nearby natural stream) was changed on alternate days. Food was withdrawn 24 h prior to experiment. Three to five fishes were used for each set of experiment.

2.2 *Liver perfusion technique*

The fish was anaesthetized in neutralized 3-aminobenzoic acid ethyl ester (MS222, 0·2 g/l) for 5 min before the operation to perform the perfusion of the liver. The liver was perfused via the portal vein in a non-circulating manner with a hemoglobin-free medium following Saha *et al* (1995). The iso-osmotic medium (265 mOsmol/l, determined by the freezing point depression method) contained 119 mM NaCl, 5 mM $NaHCO_3$, 5·4 mM KCl, 0·35 mM Na_2HPO_4, 0·44 mM KH_2PO_4, 0·81 mM $MgSO_4$ and 1·25 mM $CaCl_2$. The medium was gassed with O_2/CO_2 (99 : 1, v/v) and its pH adjusted to 7·5. The liver was perfused at a flow rate of 4 to 5 ml/g liver/min. The temperature of the medium was 30°C. The hypo-osmotic medium (– 80 mOsmol/l) was prepared by removing an equivalent amount of NaCl, and the hyper-osmotic medium (+ 80 mOsmol/l), by adding extra NaCl to the standard perfusion medium as mentioned above.

To study the rates of gluconeogenic efflux, livers were initially perfused for 20 min with iso-osmotic medium, followed by infusion of several gluconeogenic substrates (as listed in table 1) for 20 min at a concentration of 1, 2, 5 and 10 mM in different sets; 5 mM concentration was found to be most suitable for studying the gluconeogenic efflux. The effluents were collected at 2 min intervals for the determination of glucose efflux from the liver and the steady-state efflux of glucose, obtained between 16 to 20 min of infusion of the substrates, was used to calculate the rate of gluconeogenic efflux. A continuous efflux of glucose normally occurs from the perfused liver while perfusing with iso-osmotic medium (Goswami and Saha 1998). Therefore, the rate of gluconeogenic efflux was calculated by subtracting the value of steady-state efflux of glucose, obtained just before infusion, from the value of total efflux obtained after infusion of gluconeogenic substrates. Quinolinate (5 mM), a potent inhibitor of cytosolic phosphoenolpyruvate carboxykinase (PEPCK, Veneziale *et al* 1967), was also infused in different sets of experiment

to assess whether or not the extra glucose in the effluent was due to gluconeogenic activity. The pH of the final perfusion medium was always adjusted to 7·5 after addition of different substrates and/or inhibitors.

To study the effect of anisotonicity on gluconeogenesis, livers were initially perfused with iso-osmotic medium for 20 min, followed by infusion of hypo- or hyper-osmotic medium for 60 min, and then with gluconeogenic substrates for 20 min along with the aniso-osmotic medium. Anisotonicity is known to cause changes of glucose efflux from the perfused liver (Goswami and Saha 1998). Therefore, the efflux of glucose under aniso-osmotic conditions was calculated by subtracting the value of steady-state efflux of glucose just before infusion from the total efflux of glucose between 16 to 20 min of infusion of gluconeogenic substrates.

To study the effect of anisotonic exposure on the activities of gluconeogenic enzymes, livers were initially perfused with isotonic medium, followed by infusion of either hypo- (– 80 mOsmol/l) or hyper-osmotic (+ 80 mOsmol/l) medium for 3 h. During the perfusion, a small lobe of the perfused liver was cut after 1 h of infusion, and the remainder liver dropped into liquid nitrogen after 3 h, and stored at – 60°C until assayed for gluconeogenic enzymes. Similar perfusion experiments were also performed with 25 μM cycloheximide, that is sufficient to cause the inhibition of protein synthesis under perfusion conditions (Stoll *et al* 1992). Another two sets of livers were perfused for 200 min with or without cycloheximide under isotonic condition and served as controls. For determination of water content in the liver during iso-, hypo- and hypertonic perfusion conditions, another three sets of liver were used separately.

Table 1. Gluconeogenic efflux* (μmol/g liver/h) from different gluconeogenic substrates (5 mM) both in the absence or presence of an inhibitor, quinolinate (5 mM) by the perfused liver of *C. batrachus*.

Substrates	Gluconeogenic efflux (μmol/g liver/h)
Lactate	23·7 ± 0·71
Lactate + quinolinate	14·2 ± 0·9 (– 40)
Pyruvate	20·4 ± 0·28
Pyruvate + quinolinate	13·5 ± 0·8 (– 44)
L-glutamate	38 ± 0·85
L-glutamate + quinolinate	19·6 ± 1·2 (– 48)
L-glutamine	11·2 ± 0·45
L-glutamine + quinolinate	5·6 ± 0·37 (– 50)
L-alanine	9·3 ± 0·15
L-alanine + quinolinate	4·1 ± 0·6 (– 56)
L-ornithine	19·2 ± 1·8
L-ornithine + quinolinate	10·4 ± 0·7 (– 46)
L-serine	19·7 ± 0·8
L-serine + quinolinate	10·0 ± 0·8 (– 49)
L-proline	13·0 ± 1·61
L-proline + quinolinate	6·7 ± 0·6 (– 48)
L-glycine	10·5 ± 0·6
L-glycine + quinolinate	5·0 ± 0·5 (– 52)
L-aspartate	4·0 ± 0·5
L-aspartate + quinolinate	2·2 ± 0·3 (– 45)

Values are expressed as mean ± SEM (n = 3–5).
*The steady state efflux glucose from the perfused liver in the absence of gluconeogenic substrates was 67·20 ± 1·21 (n = 12) and 65·45 ± 1·55 (n = 12) μmol/g liver/h, in the absence and presence of quinolinate, respectively.

Percentage decrease of gluconeogenic efflux in the presence of inhibitor is given in parentheses.

2.3 *Estimations*

For estimation of glucose efflux from the perfused liver, 1 ml of the effluent was collected at 2 min intervals. To each sample 10 μl of 2 M perchloric acid (PCA) was added, the precipitated protein removed by centrifugation, and the supernatant neutralized by adding 10 μl of 2 M NaOH before estimation of glucose. Concentration of glucose in the effluent was measured enzymatically following the method of Bergmeyer *et al* (1974). The reaction mixture in a final volume of 1 ml contained 75 mM triethanol amine buffer (pH 7·6), 7·5 mM $MgCl_2$, 0·6 mM $NADP^+$, 1 mM Na-ATP, 0·3 unit of glucose 6-phosphate dehydrogenase (G6PDH), 0·6 unit of hexokinase and 200 μl effluent. The reaction mixture was incubated at 37°C for 30 min and the absorbance was measured at 340 nm against a control. The concentration of glucose, which was equivalent to the amount of $NADP^+$ reduced, was calculated taking $6{\cdot}22 \times 10^6$ as the molar extinction coefficient value for NADPH.

2.4 *Tissue processing*

Fish were killed by decapitation; liver, kidney, muscle and brain were excised, dipped into liquid nitrogen and stored at – 60°C. A 10% homogenate (w/v) of each frozen tissue was prepared in a homogenizing buffer containing 50 mM Tris-HCl (pH 7·4), 0·25 M sucrose, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT and 3 mM 2-mercaptoethanol. The homogenization was done with a motor driven Potter-Elvehjem type glass homogenizer fitted with a Teflon pestle. The homogenate was treated with 0·5% Triton X-100 in 1 : 1 ratio for 30 min, followed by mild sonication for 30 s. While assaying the enzyme activity in the perfused liver under hypo- and hypertonic conditions, the homogenizing buffer contained the same constituents as mentioned above but the osmolarity was adjusted by

either withdrawing or adding equivalent amount of sucrose in the medium. Triton X-100 treatment (0·5%) was also done in the same homogenizing buffer. The homogenate was then centrifuged at 10,000 *g* for 10 min and the supernatant was used for assaying the enzymes. All steps were carried out at 4°C.

2.5 *Subcellular fractionation*

Subcellular fractionation was carried out by differential centrifugation method following Dkhar *et al* (1991) with certain modifications as mentioned by Saha *et al* (1999).

2.6 *Enzyme assays*

PEPCK was assayed following Mommsen *et al* (1985) with a two-step enzymatic reaction. One ml of reaction mixture contained 50 mM Tris-HCl buffer (pH 7·4), 4·5 mM phosphoenolpyruvate, 20 mM $NaHCO_3$, 0·6 mM deoxyGDP, 0·15 mM NADH, 1 mM $MnCl_2$, 5 units of malate dehydrogenase (MDH) and 20 µl tissue extract.

Pyruvate carboxylase (PC) was assayed following Foster and Moon (1986) with a two-step enzymatic reaction. One ml of reaction mixture contained 100 mM Tris-HCl buffer (pH 7·8), 0·1 mM acetyl CoA, 15 mM $NaHCO_3$, 10 mM pyruvate, 0·15 mM NADH, 5 mM $MgCl_2$, 5 units MDH and 20 µl tissue extract.

Fructose 1,6-bisphosphatase (FBPase) was assayed following Mommsen *et al* (1985) with a three-step enzymatic reaction. One ml of reaction mixture contained 50 mM Tris-HCl buffer, 15 mM $MgCl_2$, 0·3 mM $NADP^+$, 10 units G6PDH, 400 units phosphoglucose isomerase, 0·15 mM fructose 1, 6-biphosphate and 50 µl tissue extract.

Glucose-6-phosphatase (G6Pase) was assayed following the method of Nordlie and Arion (1966). One ml of reaction mixture contained 100 mM Na-acetate buffer (pH 6·5), 40 mM glucose 6-phosphate and 50 µl tissue extract. The reaction was stopped by adding 0·5 ml of 10% trichloroacetic acid after a specific period of time. The inorganic phosphate formed was estimated in the supernatant spectrophotometrically at 700 nm following Fiske and Subbarow (1957) against a tissue blank, and expressed as enzyme activity.

Lactate dehydrogenase (LDH) was assayed following Vorhaben and Campbell (1972). One ml of reaction mixture contained 30 mM Na-phosphate buffer (pH 7·4), 5 mM pyruvate, 0·2 mM NADH and 50 µl tissue extract.

Glutamate dehydrogenase (GDH) was assayed following Olson and Anfinsen (1952) with certain modifications made by Das *et al* (1991). One ml of reaction mixture contained 100 mM K-phosphate buffer (pH 8·5), 50 mM NH_4Cl, 25 mM *a*-ketoglutarate, 0·3 mM NADH, 1·0 mM ADP, 0·2 mM EDTA and 50 µl tissue extract.

The decrease in absorbance (due to oxidation of NADH to NAD^+ in the case of PEPCK, PC, LDH and GDH), and the increase in absorbance (due to reduction of $NADP^+$ to NADPH in the case of FBPase) were recorded at 30 s interval at 340 nm in a uv-visible spectrophotometer (Beckman, Model DU 640) fitted with a peltier temperature-controlled device. One unit of enzyme activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 µmol of NADH per min in the case PEPCK, PC, LDH and GDH, or the reduction of 1 µmol of $NADP^+$ per min in the case of FBPase at 30°C. For G6Pase, one unit of enzyme activity was expressed as that amount of enzyme which catalyzed the formation of 1 µmol of inorganic phosphate per min at 30°C.

2.7 *Measurement of water content in the perfused liver*

The water content in the perfused liver under different experimental conditions was determined by oven drying following Goswami and Saha (1998).

2.8 *Chemicals*

Enzymes, co-enzymes, substrates and MS222 were purchased either from Sigma Chemical Co. (St. Louis, USA) or Roche Applied Science (Germany). Other chemicals were of analytical grades and were obtained from local sources. Deionized double-glass-distilled water was used in all preparations.

2.9 *Statistical analysis*

Data collected from different replicates were statistically analysed and presented as mean ± SEM (*n*), where *n* equals the number of animals in the sample. One-way ANOVA test was performed to compare the experimental values with those of respective controls. Differences at $P < 0·05$ were regarded as statistically significant.

3. Results

3.1 *Gluconeogenic efflux by the perfused liver*

The data on the gluconeogenic efflux under the influence of the various gluconeogenic substrates is depicted in table 1. The results indicate that in addition to lactate and pyruvate, some amino acids also served as potential gluconeogenic substrates in the perfused liver of *C. batrachus*. The rate of gluconeogenesis was highest with glutamate and least with aspartate, being in the order of glutamate > lactate, pyruvate > serine > ornithine > proline > glutamine > glycine > alanine > aspartate. The gluconeogenic efflux from dif-

ferent substrates was inhibited by 40% to 50% when quinolinate (5 mM) was infused along with the test substrates (table 1).

3.2 *Physiological levels of activity of gluconeogenic enzymes*

As shown in table 2, the physiological activities of PEPCK, PC, FBPase and G6Pase were found to be more in liver and kidney, while the first mentioned three enzymes showed relatively low levels in muscle and brain; G6Pase activity could not be detected in these tissues.

Table 2. The levels of activity (units/g wet wt.) of four key gluconeogenic enzymes in different tissues of *C. batrachus*.

	Tissues			
Enzymes	Liver	Kidney	Muscle	Brain
PEPCK	8·77 ± 0·80	2·75 ± 0·13	1·1 ± 0·05	2.43 ± 0.10
PC	31·00 ± 1·70	10·21 ± 1·12	0.46 ± 0.07	9.87 ± 0.20
FBPase	9·25 ± 0·34	2·34 ± 0·30	1.34 ± 0.07	0.67 ± 0.10
G6Pase	5·21 ± 0·25	0·98 ± 0·09	BLD	BLD

Values are expressed as mean ± SEM (n = 5). One unit of enzyme activity was expressed as 1 μmol of NADH oxidized in the case of PEPCK and PC, and 1 μmol of $NADP^+$ reduced in the case of FBPase per min at 30°C. For G6Pase, one unit of enzyme activity is expressed as that amount which catalyzed the formation of 1 μmol of inorganic phosphate per min at 30°C.
BLD, below the level of detection.

3.3 *Subcellular distribution of gluconeogenic enzymes in the liver*

The subcellular distribution of PEPCK, PC and FBPase was studied in the liver of *C. batrachus* (table 3). For PEPCK, about 45% of activity was in mitochondria and 50% in the cytosol. For PC, 93% of activity was in mitochondria, with very little activity in the cytosol. 66% of FBPase activity was localized in the mitochondria and 42% in the cytosol. This was accompanied by 79% of GDH activity in the mitochondrial fraction and 95% of LDH activity in the cytosolic fraction, thus indicating that the mitochondrial separation was proper. It is evident from these results that PC is exclusively localized in mitochondria, and PEPCK and FBPase are localized both in the mitochondria and cytosol.

3.4 *Effects of anisotonicity on the hydration status of hepatocytes*

As shown in table 5, the water content of hepatocytes under iso-osmotic perfusion condition averaged to about 77%, which was not affected by the presence of cycloheximide. However, the hypo-osmotic insult (– 80 mOsmol/l) caused a significant increase ($P < 0·01$) in water content of hepatocytes by 9·7% after 1 h, whereas, the hyperosmotic insult (+ 80 mOsmol/l) caused a significant decrease ($P < 0·01$) in water content by 12·3% after 1 h, and this increase or decrease was maintained till 3 h of perfusion. Changes in hydration status of hepatocytes due to anisotonicity remained unaltered in the presence of cycloheximide.

Table 3. Subcellular distribution of different gluconeogenic enzymes in the liver of *C. batrachus*.

Enzymes	Homogenate	Nuclear	Mitochondrial	Cytosolic	Recovered activity
PEPCK	11.02 ± 0.57	1.03 ± 0.12 (9)	5.0 ± 0.34 (45)	5.5 ± 0.45 (50)	11.53 ± 0.81 (105)
PC	32.17 ± 2.81	1.26 ± 0.06 (4)	30.0 ± 3.06 (93)	4.6 ± 0.45 (14)	35.85 ± 3.41 (111)
FBPase	7.29 ± 0.89	0.35 ± 0.02 (14)	4.80 ± 0.72 (66)	3.09 ± 0.33 (42)	8.24 ± 1.62 (113)
GDH	112 ± 8.12	3.48 ± 0.23 (3)	88.23 ± 3.42 (79)	10.40 ± 0.37 (9)	102.11 ± 7.81 (91)
LDH	45 ± 3.42	2.33 ± 0.14 (5)	4.12 ± 0.37 (9)	42.61 ± 3.41 (95)	49.06 ± 4.64 (109)

Values are expressed as mean ± SEM (n = 3). GDH and LDH were used as marker enzymes for the mitochondrial and cytosolic fractions, respectively.

Percentage of enzyme activity in different fractions compared to the total activity in the homogenate is given in parentheses.

One unit of LDH and GDH activities was expressed as that amount of enzyme which catalyzed the oxidation of 1 μmol of NADH to NAD^+ per min at 30°C.

3.5 *Effects of anisotonicity on gluconeogenic efflux by the perfused liver*

Effects of anisotonicity on gluconeogenesis in the perfused liver of *C. batrachus* were studied only with lactate, pyruvate and glutamate, since maximum gluconeogenic efflux was obtained with these substrates (table 4). For all the three substrates, the gluconeogenic efflux decreased significantly when the liver was perfused with the hypo-osmotic (– 80 mOsmol/l) medium. Opposing effects were seen when the liver was perfused with the hyper-osmotic (+ 80 mOsmol/l) medium.

The rates of gluconeogenesis from lactate, pyruvate and glutamate were also studied in iso- and aniso-osmotic conditions in the presence of cycloheximide in order to assess the possible involvement of new enzyme protein synthesis, thereby changing the rates of gluconeogenesis under aniso-osmotic conditions (table 4). Cycloheximide (25 μM) as such did not cause any significant change on gluconeogenic efflux under iso-osmotic condition. However, the inhibitory effects of hypotonicity on gluconeogenic efflux with the three substrates were partly blocked in the presence of cycloheximide as were also the stimulatory effects of hypertonicity (table 4).

3.6 *Effects of anisotonicity on the activity of gluconeogenic enzymes in the perfused liver*

The changes of activity of the four key gluconeogenic enzymes in the perfused liver of *C. batrachus* were studied after 1 and 3 h of perfusion with aniso-osmotic media both in the presence or absence of cycloheximide (table 5). No significant changes of activity of these enzymes were observed when the liver was perfused for 3 h with iso-osmotic medium in the absence or presence of cycloheximide. Under hypo-osmotic perfusion condition, the activities of PEPCK, PC and G6Pase decreased significantly ($P < 0{\cdot}01$) after 1 h, the maximum percentage decrease being 40, 53 and 41%, respectively, though the activity of FBPase remained unaltered even after 3 h. In the presence of cycloheximide, the inhibitory effect of hypotonicity on the activities of PEPCK and PC was partly abolished, but persisted with respect to G6Pase.

Under hyper-osmotic perfusion condition opposing effects were noticed, i.e. a significant increase in the activities of PEPCK ($P < 0{\cdot}05$), FBPase ($P < 0{\cdot}05$) and G6Pase ($P < 0{\cdot}01$) after 1 h, followed by a further increase after 3 h (table 5). The maximum percentage increase of activity of PEPCK, PC, FBPase and G6Pase was 67, 40, 78

Table 4. Gluconeogenic efflux (μmol/g liver/h) from important gluconeogenic substrates by the perfused liver of *C. batrachus* under aniso-osmotic (+/– 80 mOsmol/l) conditions.

Substrates	Conditions	Gluconeogenic efflux (μmol/g liver/h)	Percentage changes
Lactate (5 mM)	Iso-osmotic	23.7 ± 0.7	
	Hypo-osmotic	8.4 ± 0.6[c]	– 65
	Hyper-osmotic	29.3 ± 2.9[a]	+ 24
Lactate (5 mM) + cycloheximide (25 μM)	Iso-osmotic	21.8 ± 1.25	
	Hypo-osmotic	16.7 ± 0.81[b]	– 24
	Hyper-osmotic	25.8 ± 1.52	+ 9
Pyruvate (5 mM)	Iso-osmotic	20.4 ± 1.21	
	Hypo-osmotic	7.8 ± 0.4[c]	– 62
	Hyper-osmotic	25.9 ± 2.1[a]	+ 27
Pyruvate (5 mM) + cycloheximide (25 μM)	Iso-osmotic	22.1 ± 1.61	
	Hypo-osmotic	18.8 ± 0.58	– 15
	Hyper-osmotic	24.6 ± 1.20	+ 11
Glutamate (5 mM)	Iso-osmotic	38.0 ± 1.81	
	Hypo-osmotic	17.8 ± 2.20[c]	– 55
	Hyper-osmotic	52.5 ± 3.80[b]	+ 38
Glutamate (5 mM) + cycloheximide (25 μM)	Iso-osmotic	35.4 ± 2.12	
	Hypo-osmotic	30.4 ± 2.31	– 14
	Hyper-osmotic	40.7 ± 3.12	+ 15

Values are expressed as mean ± SEM ($n = 3$). [a,b,c]Significantly different from iso-osmotic control values at $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels, respectively (one-way ANOVA test).

Table 5. Effects of anisotonicity on the activity of different gluconeogenic enzymes in the liver of *C. batrachus* after 1 and 3 h of perfusion.

	After 1 h					After 3 h				
Conditions	PEPCK	PC	FBPase	G6Pase	Percentage of water content	PEPCK	PC	FBPase	G6Pase	Percentage of water content
Iso-osmotic	11.93 ± 0.95	30.75 ± 2.73	4.23 ± 0.33	3.64 ± 0.34	77 ± 2.2	15.33 ± 1.14	30.33 ± 2.45	4.82 ± 0.1	3.51 ± 0.19	77.5 ± 2.3
+ cycloheximide (25 μM)	11.00 ± 0.88	27.5 ± 1.87	3.54 ± 0.21	3.55 ± 0.39	76 ± 2.4	12.51 ± 1.32	28.07 ± 2.21	3.81 ± 0.09	3.45 ± 0.24	77.0 ± 2.1
Hypo-osmotic (– 80 mOsmol l^{-1})	7.15 ± 2.12[a] (– 40)	14.25 ± 3.1[b] (– 53)	4.37 ± 0.64 (– 3)	2.15 ± 0.28[b] (– 41)	84.5 ± 2.7[b] (+ 9.7)	11.03 ± 0.45[a] (– 28)	23.33 ± 1.03[a] (– 23)	4.70 ± 0.17 (– 2)	1.97 ± 0.17[c] (– 44)	85.0 ± 2.4[a] (+ 9.6)
+ cycloheximide (25 μM)	9.74 ± 3.12 (– 11)	23.80 ± 2.53 (– 13)	3.66 ± 0.51 (+ 3)	1.90 ± 0.21[b] (– 46)	84.0 ± 1.9[a] (+ 10)	10.45 ± 0.33 (– 16)	25.78 ± 1.33 (– 8)	3.72 ± 0.10 (+ 3)	1.87 ± 0.19[c] (– 46)	84.5 ± 2.1[a] (+ 9.7)
Hyper-osmotic (+ 80 mOsmol l^{-1})	15.15 ± 1.0[a] (+ 27)	34 ± 1.38 (+ 10)	6.61 ± 0.62[a] (+ 56)	6.11 ± 0.41[b] (+ 67)	67.5 ± 2.3[b] (– 12.3)	25.67 ± 2.36[a] (+ 67)	42.67 ± 0.55[b] (+ 40)	8.59 ± 1.15[b] (+ 78)	6.29 ± 0.32[c] (+ 80)	68.0 ± 1.8[b] (– 12)
+ cycloheximide (25 μM)	12.54 ± 1.0 (+ 14)	30.53 ± 1.10 (+ 11)	4.08 ± 0.38 (+ 15)	5.86 ± 0.36[b] (+ 65)	67.0 ± 1.5[a] (– 12)	14.12 ± 1.85 (+ 13)	32.12 ± 1.61 (+ 14)	4.27 ± 0.38 (+ 12)	6.05 ± 0.36[c] (+ 76)	68.5 ± 1.6[b] (– 11)

Values are expressed as mean ± SEM (n = 3). Percentage increase (+)/decrease (–) of enzyme activity or water content under aniso-osmotic conditions compared to iso-osmotic controls are given in parentheses.

[a,b,c]Significantly different from iso-osmotic control values at $P < 0{\cdot}05$, $P < 0{\cdot}01$ and $P < 0{\cdot}001$ levels, respectively (one-way ANOVA test).

and 80%, respectively, after 3 h of hyper-osmotic perfusion. The stimulatory effects of hypertonicity on the activities of PEPCK, PC and FBPase were also partly abolished, but not so for G6Pase when the liver was perfused in the presence of cycloheximide for 3 h.

4. Discussion

C. batrachus is predominantly a carnivorous fish and primarily depends on high protein and low carbohydrate diets (Munshi and Ghosh 1994). Therefore, it seemed of interest to study the gluconeogenic activity in this fish. As evidenced from the results of the present study, apart from lactate and pyruvate, some amino acids such as glutamate, alanine, glutamine, ornithine, serine, proline and glycine can serve as good gluconeogenic substrates in the liver of the catfish with a maximal activity with glutamate. This suggests that dietary amino acids and also the amino acids endogenously produced via proteolysis during starvation can serve as major gluconeogenic substrates for the maintenance of plasma glucose, even though the concentration of amino acids infused here in the perfused liver was above the physiological concentration (Saha *et al* 2002). A higher prevalence of amino acid gluconeogenesis, rather than lactate gluconeogenesis, is reported to occur in skates (Mommsen and Moon 1987) and in skipjack tuna (Buck *et al* 1992), possibly because of having an inactive Cori cycle. Renaud and Moon (1980b), however, emphasized the importance of lactate gluconeogenesis rather than amino acid gluconeogenesis in American eel (*Anguilla rostrata*) during starvation. The importance of amino acid and lactate gluconeogenesis in the walking catfish may be further clarified by studying the gluconeogenic rates from different substrates after a prolonged starvation, since the hepatic carbohydrate stores in fish may remain constant for several months (Dave *et al* 1975) unlike mammals, in which they are depleted within 48 h (Ross *et al* 1967). There could be many reasons for variations of gluconeogenic efflux obtained with different substrates in the catfish, for example differences in the efficiency of transport of substrates into the hepatocytes, or different rates of transamination reactions of amino acids for ultimately entering into the gluconeogenic pathway, as suggested by Walton and Cowey (1979) in rainbow trout. It seems likely that aspartate has a low permeability through the hepatocyte membrane, resulting in very low rate of gluconeogenesis in the fish under the present study. Subcellular compartmentation of different enzymes could be another possible reason for the different rates of gluconeogenic efflux with different substrates. It is difficult to compare the rate of gluconeogenesis observed in this catfish with other teleost species, since the process was studied in the whole liver organ by the perfusion technique, which we believe to be more physiological rather than studying in liver slices or in isolated cells. Nonetheless, it is evident from our results that gluconeogenesis both from lactate as well as from amino acids is very active in this catfish, and is certainly higher than in carps and other teleosts (Janssens and Waterman 1988; Moon *et al* 1985), though comparable with Japanese eel (Hayashi and Ooshiro 1979) and some carnivorous fishes (Walton and Cowey 1979; Renaud and Moon 1980a,b; Moon *et al* 1985; Peterson *et al* 1987). Presence of high levels of activity of gluconeogenic enzymes in the liver and kidney of *C. batrachus* as compared to many teleosts (for review, see Moon and Foster 1995) confirms that these two are the major gluconeogenic organs in this fish, a situation that is similar to that observed in other fish species such as rainbow trout, cod and plaice (Knox *et al* 1980; Panserat *et al* 2001).

A low rate of gluconeogenesis from amino acids, as reported in most teleosts, is suggested to be mainly due to the mitochondrial localization of PEPCK enzyme in the liver (for review, see Moon *et al* 1985). However, in *C. batrachus* liver, both PEPCK and FBPase are equally distributed both in the mitochondria and cytosol, similar to the situation found in Japanese eel (Hayashi and Ooshiro 1979). However, in mammals, FBPase is primarily localized in the cytosol (Söling and Kleineke 1976). The equal distribution of PEPCK as mitochondrial and cytosolic in *C. batrachus* hepatocytes is further supported by the observation that 50% of gluconeogenic activity in the perfused liver was inhibited by the presence of quinolinate (table 1); the latter is known to inhibit only the cytosolic form of PEPCK activity (Söling *et al* 1970); compartmentation of gluconeogenic enzymes in the fish may be of regulatory significance as reported for PEPCK in other fishes such as in plaice (*Pleuronectes platessa*) (Moon and Johnston 1980) and in chicken (Weldon *et al* 1990). Further, it may be crucial to the understanding of the effects of mitochondrial and cytosolic redox potential on overall gluconeogenic fluxes and apparent substrate preferences.

The effect of various environmental factors such as temperature, hypoxia, starvation, and certain hormones on carbohydrate metabolism including gluconeogenesis in various fish species has been reported by several workers (for review, see Moon and Foster 1995). Besides, anisoosmotic exposure is another major environmental factor which can influence the carbohydrate metabolism in walking catfish (Goswami and Saha 1998; Saha and Goswami 2004). Hypo-osmotic cell swelling is reported to cause a decrease in the production of glucose, lactate and pyruvate from the perfused liver of catfish with a decrease and increase of activities of glycogen phosphorylase and glycogen synthase enzymes, respectively, but opposing effects were observed under hyper-osmotic cell shrinkage (Goswami and Saha 1998). As observed in the present

study, gluconeogenesis is also affected by anisotonicity at least in the liver, thus causing a decrease and increase in the rates of gluconeogenic efflux by the perfused liver under hypo-osmotic cell swelling and hyper-osmotic cell shrinkage, respectively, with all the three substrates (lactate, pyruvate and glutamate). This was accompanied with favourable changes of activity of some gluconeogenic enzymes. Hypo-osmotic cell swelling resulted in a significant decrease of activity of PEPCK, PC and G6Pase, and hyper-osmotic cell shrinkage resulted in a significant increase of PEPCK, PC, FBPase and G6Pase activities. These osmosensitive changes of gluconeogenic efflux along with the changes of activity of related enzymes, obtained in this fish, were likely due to changes of hydration status of the hepatocytes, which remained so as long as they were exposed to aniso-osmotic conditions. This is in contrast to the situation in some other teleosts, in which a transient change of hepatocyte cell volume occurs during anisotonic exposure, and the original cell volume is re-established within few minutes (Bianchini *et al* 1988, 1991; Michel *et al* 1994; Jackson *et al* 1996).

The hyper- and hypo-osmotic treatments do not compromise with the cell integrity as judged by the unaltered leakage of cytosolic LDH into the effluent from the perfused liver, which varied between 1 to 2 units/l all through the experiment both in iso- and anisotonic conditions (results not shown). Therefore, we may conclude that the noted effects are indeed attributable to changes of the cell volume and not to the partial physical damage to the cells.

There could be various possible mechanism(s) of regulation of gluconeogenic efflux during cell volume changes in the hepatocytes of this catfish. One such mechanism could be regulation of the synthesis of new protein(s) during cell volume changes, since the stimulatory and inhibitory effects of anisotonicity on gluconeogenesis as well as the enzymic activities were found to be partially blocked by the presence of cycloheximide. Quillard *et al* (1998) demonstrated that the decreasing effect of hypotonicity on gluconeogenesis in the rat hepatocyte requires the synthesis of a 45,000 Mr protein, which could be blocked by inhibiting protein synthesis. Second, there could be a regulation by changing the phosphorylation status of the regulatory enzymes, as observed in the case of GPase and GSase enzymes in *C. batrachus* (Goswami and Saha 1998), and GPase activity in another catfish, *Amereiurus nebulosus* (Hallgren *et al* 2003). Numerous protein kinases have been implicated in metabolic alterations with volume changes in the mammalian hepatocytes (Weiergräber and Häussinger 2000) and the fish chloride cells (Kültz and Avila 2001), and several osmosensing receptors responsible for activation of various kinases have been identified (Maeda *et al* 1995). Molecular crowding may also exert long-term effects, such as cAMP-mediated changes in gene transcription (Burg 2000; Minton 2001), since the hydration status of hepatic cells of this catfish remains altered throughout the period of osmotic insult. Therefore, a thorough investigation on the aspects of metabolic regulation of hepatocytes with relation to cell volume changes is required before any definite conclusion could be drawn.

In conclusion, gluconeogenesis appears to play a vital role in this air-breathing walking catfish for maintaining the glucose homeostasis and also for high glucose production mostly from dietary proteins and amino acids as may be required for proper energy supply mainly to support metabolic demands for ion transport and other metabolic activities during cell volume changes under osmotic and other environmental stresses encountered in their natural habitat.

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