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# Effects of anisotonicity on pentose-phosphate pathway, oxidized glutathione release and t-butylhydroperoxide-induced oxidative stress in the perfused liver of air-breathing catfish, *Clarias batrachus*

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Both hypotonic exposure (185 mOsmol/l) and infusion of glutamine plus glycine (2 mmol/l each) along with the isotonic medium caused a significant increase of  $^{14}\text{CO}_2$  production from [1- $^{14}\text{C}$ ]glucose by 110 and 70%, respectively, from the basal level of  $18.4 \pm 1.2$  nmol/g liver/min from the perfused liver of *Clarias batrachus*. Conversely, hypertonic exposure (345 mOsmol/l) caused significant decrease of  $^{14}\text{CO}_2$  production from [1- $^{14}\text{C}$ ]glucose by 34%.  $^{14}\text{CO}_2$  production from [6- $^{14}\text{C}$ ]glucose was largely unaffected by anisotonicity. The steady-state release of oxidized glutathione (GSSG) into bile was  $1.18 \pm 0.09$  nmol/g liver/min, which was reduced significantly by 36% and 34%, respectively, during hypotonic exposure and amino acid-induced cell swelling, and increased by 34% during hypertonic exposure. The effects of anisotonicity on  $^{14}\text{CO}_2$  production from [1- $^{14}\text{C}$ ]glucose and biliary GSSG release were also observed in the presence of t-butylhydroperoxide (50  $\mu\text{mol/l}$ ). The oxidative stress-induced cell injury, caused due to infusion of t-butylhydroperoxide, was measured as the amount of lactate dehydrogenase (LDH) leakage into the effluent from the perfused liver; this was found to be affected by anisotonicity. Hypotonic exposure caused significant decrease of LDH release and hypertonic exposure caused significant increase of LDH release from the perfused liver. The data suggest that hypotonically-induced as well as amino acid-induced cell swelling stimulates flux through the pentose-phosphate pathway and decreases loss of GSSG under condition of mild oxidative stress; hypotonically swollen cells are less prone to hydroperoxide-induced LDH release than hypertonically shrunken cells, thus suggesting that cell swelling may exert beneficial effects during early stages of oxidative cell injury probably due to swelling-induced alterations in hepatic metabolism.

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## 1. Introduction

The hepatocellular hydration state is now recognized as an important physiological determinant of cell function and that hormones, nutrients, amino acids and oxidative stress exert their effects on metabolism and gene expression in liver in part by a modification of cell volume (for review, see Häussinger 1996). Cell volume is frequently

being challenged either due to intestinal absorption of water and nutrients and/or due to exposure to different osmotic environments especially in case of aquatic animals.

Most normal cells can regulate their cell volume under anisotonic conditions, thus osmotic swelling and shrinkage are ordinarily followed by regulatory volume decrease (RVD) and regulatory volume increase (RVI), respectively (Hoffman and Simonsen 1989; Häussinger and Lang 1991).

**Keywords.** Anisotonicity; cell injury; cell volume; *Clarias batrachus*; GSSG; oxidative stress; pentose-phosphate pathway; perfused liver; t-butylhydroperoxide

Abbreviations used: GSH, Glutathione; GSSG, oxidized glutathione; LDH, lactate dehydrogenase; RVD, regulatory volume decrease; RVI, regulatory volume increase; t-BOOH, t-butylhydroperoxide.

However, it should be noted that the initial cell volume is not restored completely following RVD or RVI in most of the cell types, i.e. the cells remain either in a slightly swollen or shrunken state for the duration of the anisotonic exposure, and this slight changes of cell volume or hydration status of liver cells are reported to cause lots of metabolic changes including that of carbohydrate and lipid metabolism, proteolysis and protein synthesis, taurocholate excretion into bile, release of reduced glutathione (GSH) into the effluent and oxidized glutathione (GSSG) into bile, and also oxidative stress-induced cell damage in perfused rat liver (for review, see Häussinger 1996). Cell shrinkage has also been reported in apoptotic cells (Okada and Maeno 2001) and during oxidative stress (Hallbrucker *et al* 1993; Saha *et al* 1993) even under isotonic conditions.

In comparison to mammals, teleost fishes face more problems of osmotic stress primarily due to osmolarity changes to their external environment at different seasons of the year. Further, teleost fishes also face more problems of oxidative stress due to enhanced production of reactive oxygen species in the presence of environmental contaminants, thus causing a threat to aquatic organisms (Livingstone *et al* 1990; Winston and Di Giulio 1991). Oxidative stress, resulted due to intracellular generation of hydrogen peroxide, is reported to cause cell shrinkage by opening of  $K^+$  channel and more efflux of GSSG from the perfused rat liver (Saha *et al* 1993). Further, it has been suggested that cellular shrinkage in skeletal muscle and liver in response to a variety of mediators of inflammation, including oxidative stress, might be one of the important triggers for the changes of protein catabolic state (Häussinger *et al* 1993). Various studies have been carried out in fishes regarding the mechanisms of cell volume regulation (Bianchini *et al* 1988; Jensen 1995; Perlman *et al* 1996), but few informations are available on alterations of different metabolic processes in relation to cell volume changes. More recently, the effects of cell volume changes on glycogen metabolism (Goswami and Saha 1998) and on gluconeogenesis (C Goswami, S Datta, K Biswas and N Saha, Unpublished results) have been reported in the perfused liver of air-breathing catfish, *Clarias batrachus*.

Compared to typical freshwater teleosts, certain air-breathing fishes from Indian subcontinent, which live normally in slow flowing, stagnant and polluted water bodies of ponds, lakes and swamps, are reported to be more resistant to environmental changes (for review, see Saha and Ratha 1998). Because of the type of habitat they occupy, these fishes regularly face problems associated with osmolarity changes and oxidative stress mainly due to the presence of various contaminants into their natural habitat, besides other environmental constrains like high ambient ammonia, desiccation and alkalinity stresses etc. in different seasons of the year.

t-Butylhydroperoxide (t-BOOH) is frequently being used as a model compound for studies on oxidative stress on liver cells (Sies 1985). In rat liver, t-BOOH is reported to reduce to t-butanol by glutathione peroxidase, but is not a substrate for catalase, and steady state rates of GSSG release into bile, which is normally used as a good indicator for oxidative stress in perfused rat liver (Sies 1985), can be achieved upon infusion of t-BOOH. Here we report the effects of anisotonicity on pentose-phosphate pathway, GSSG release into bile, relative rates of cell injury caused due to t-BOOH-induced oxidative stress in the perfused liver of air-breathing catfish, *C. batrachus*.

## 2. Materials and methods

### 2.1 Animals

The fish, *C. batrachus*, weighing 60–100 g were purchased from commercial sources and acclimatized in the laboratory at  $28 \pm 2^\circ\text{C}$  in plastic aquaria with 12 h : 12 h light and dark photoperiods before being used for experiments. Minced pork liver and rice bran (5% of body weight) was given as food on alternate days. Fishes were used after at least four weeks of acclimatization under the laboratory conditions when the mortality rate became zero and the food consumption was normal. No sex determination was done while performing the experiments.

### 2.2 Liver perfusion technique

The fish was anaesthetized in neutralized 3-aminobenzoic acid ethyl ester (MS-222, 0.2 g/l) for 2 min before operation for liver perfusion. Liver was perfused, by way of a ventral incision made on the hepatic portal vein, in a non-circulating manner in the physiological antegrade direction with a hemoglobin-free medium following Saha *et al* (1995). The perfusion medium contained 119 mM NaCl, 5 mM  $\text{NaHCO}_3$ , 5.4 mM KCl, 0.35 mM  $\text{Na}_2\text{HPO}_4$ , 0.81 mM  $\text{MgSO}_4$  and 1.25 mM  $\text{CaCl}_2$  as the basic solution for perfusion. The osmolarity of the perfusing medium was 265 mOsmol/l, since the osmolarity of blood of *C. batrachus* was found to be 265 mOsmol/l (determined by the freezing point depression method with a Camlab osmometer, Model 2000). The medium also contained 1.2 mM lactate, 0.3 mM pyruvate and 5 mM D-glucose. The perfusate was saturated with  $\text{O}_2/\text{CO}_2$  (99/1 by v/v), before infusing into the liver. The flow rate was maintained at 4–5 ml/g liver per min, the temperature was  $30^\circ\text{C}$ , and the pH of the medium was always maintained at 7.6 after gassing. The hypotonic (– 80 mOsmol/l) perfusion condition was achieved by removing equivalent amount of NaCl from the perfusion medium, and to achieve hypertonic (+ 80 mOsmol/l) perfusion condition, equivalent amount

of NaCl was added into the perfusion medium. In some of the experiments, t-BOOH, which is known to cause oxidative stress (Brigelius 1983; Sies 1985), was infused by the use of a micro precision pump to the perfused liver under iso-, hypo-, or hypertonic conditions.

### 2.3 Bile flow and estimation of GSSG into the bile and perfusate

The bile was collected from the perfused liver by inserting a narrow catheter through the major bile duct coming out from the liver and collected in pre-weighed eppendorf cups at 5 min intervals. The rate of bile flow was determined from the secreted bile mass, assuming a specific mass of 1 g/ml and expressed as  $\mu\text{l/g liver/min}$ . The GSSG in the bile and in perfusate was measured enzymatically following the method of Akerboom and Sies (1981). One ml of reaction mixture contained 100 mM K-phosphate buffer (pH 7), 1 mM EDTA, 0.4 mM NADPH, 5 units of glutathione reductase, and 5  $\mu\text{l}$  bile or 100  $\mu\text{l}$  perfusate. The reaction mixture was incubated at 37°C for 30 min to convert all the GSSG to reduced GSH, and the absorbance difference due to oxidation of NADPH to  $\text{NADP}^+$  against a control was recorded at 340 nm in a uv-visible spectrophotometer (Beckman, DU 640) and the concentration of GSSG present in the bile was calculated taking  $6.22 \times 10^6$  as molar extinction coefficient for NADPH.

### 2.4 Measurement of $^{14}\text{CO}_2$ efflux from the perfused liver

$^{14}\text{CO}_2$  production from [1- $^{14}\text{C}$ ]glucose and [6- $^{14}\text{C}$ ]glucose, and its efflux in the effluent was determined following the method of Stoll and Häussinger (1989). Perfusate, containing [1- $^{14}\text{C}$ ]glucose or [6- $^{14}\text{C}$ ]glucose (120–180 kBq/l), was pooled over 5 min periods and collected in stoppered conical flasks. Into the stoppered conical flask, 2 ml of 2 M  $\text{H}_2\text{SO}_4$  was added through a syringe to volatilize out  $^{14}\text{CO}_2$  which was trapped in 1 ml phenyl ethylamine for 18 h while keeping hanged inside the flask in a scintillation vial. The radioactivity was determined by scintillation spectrometry (Packard 1600 TR). Rates of  $^{14}\text{CO}_2$  production were calculated on the basis of the specific radioactivity of glucose in the influent perfusate.

### 2.5 Lactate dehydrogenase assay in the effluent

Effluents, coming out of the perfused liver, were collected at 5 min intervals all through the experiment and used immediately for lactate dehydrogenase (LDH) assay to assess the degree of cell damage, caused due to oxidative stress by the infusion of t-BOOH under iso- and anisotonic conditions. LDH in the effluent was assayed following the method of Vorhaben and Campbell (1972). One ml of

reaction mixture contained 50 mM Na-phosphate buffer (pH 7.4), 10 mM pyruvate, 0.4 mM NADH and 200  $\mu\text{l}$  of the effluent. The linear decrease of absorbance changes due to oxidation of NADH to  $\text{NAD}^+$  was recorded in a uv-visible spectrophotometer (Beckman, DU 640) at 340 nm at 30°C, and the LDH activity was calculated taking  $6.22 \times 10^6$  as molar extinction coefficient value for NADH. One unit of LDH activity is defined as that amount of enzyme which catalyzes oxidation of 1  $\mu\text{mol}$  of NADH to  $\text{NAD}^+$  at 30°C. The LDH release by the perfused liver was expressed as milliUnits/g liver/min.

### 2.6 Measurement of water content in the perfused liver

The water content in the perfused liver under iso- and anisotonic conditions was determined by oven drying method following Goswami and Saha (1998). Determination of hydration status of perfused liver was performed in a different set of experiment, where the perfusion was stopped either in hypotonic or hypertonic conditions instead of reperfusing back with normotonic medium.

### 2.7 Statistical analysis

Data collected from three to six replicates were statistically analysed and presented as mean  $\pm$  SEM. Comparisons of the unpaired mean values between the experimental and respective controls were made using unpaired students *t*-test and differences with  $P < 0.05$  were regarded as statistically significant.

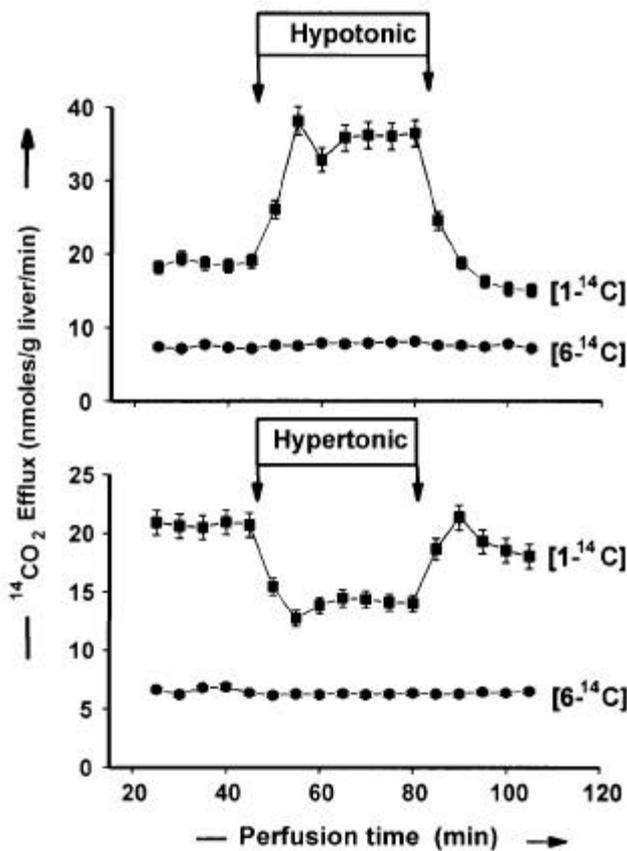
### 2.8 Chemicals

[1- $^{14}\text{C}$ ]glucose was obtained from Board of Radiation and Isotope Technology, Bhabha Atomic Research Centre (BARC), Mumbai and [6- $^{14}\text{C}$ ]glucose was from Amersham Pharmacia Biotech, UK. t-BOOH and glutathione reductase were from Sigma Chemicals Co., St. Louis, USA. All other chemicals used were of analytical grades and mostly obtained from Sisco Research Laboratory, Mumbai.

## 3. Results

### 3.1 Effects of anisotonicity on $^{14}\text{CO}_2$ production from [1- $^{14}\text{C}$ ]glucose and [6- $^{14}\text{C}$ ]glucose, bile flow and GSSG release into bile

The  $^{14}\text{CO}_2$  production from [1- $^{14}\text{C}$ ]glucose and [6- $^{14}\text{C}$ ]glucose from the perfused liver of *C. batrachus* and its efflux was measured both under iso- and anisotonic perfusion conditions (figure 1; table 1). In normotonic perfusions,  $^{14}\text{CO}_2$  produced from [1- $^{14}\text{C}$ ]glucose (5 mM) was  $18.4 \pm 1.2$  nmol/g



**Figure 1.** Effects of anisotonicity on  $^{14}\text{CO}_2$  production from  $[1\text{-}^{14}\text{C}]$ glucose and  $[6\text{-}^{14}\text{C}]$ glucose in the perfused liver of *C. batrachus*. Values are plotted as mean  $\pm$  SEM ( $n = 3$ ).

liver/min ( $n = 6$ ). Hypotonic exposure significantly stimulated the  $^{14}\text{CO}_2$  production from  $[1\text{-}^{14}\text{C}]$ glucose by 110% ( $P < 0.001$ ), whereas hypertonic exposure significantly inhibited the  $^{14}\text{CO}_2$  production by 34% ( $P < 0.05$ ). The effects of anisotonicity on  $^{14}\text{CO}_2$  production from  $[1\text{-}^{14}\text{C}]$ glucose persisted throughout hypo- and hypertonic exposures, and were fully reversible upon restoration of normotonic perfusion conditions (figure 1). On the other hand, the  $^{14}\text{CO}_2$  production from  $[6\text{-}^{14}\text{C}]$  glucose was recorded to be  $7.55 \pm 0.75$  nmol/g liver/min ( $n = 6$ ) during normotonic control perfusions and was largely unaffected by anisotonic exposure (figure 1; table 1).

As shown in figure 2 and table 1, during normotonic control perfusions the average bile flow and GSSG release into the bile from the perfused liver of *C. batrachus* were  $1.05 \pm 0.003$   $\mu\text{l/g}$  liver/min ( $n = 6$ ) and  $1.18 \pm 0.003$  nmol/g liver/min ( $n = 6$ ), respectively. No significant changes of bile flow by the perfused liver was observed during anisotonic perfusion conditions compared to isotonic controls, except for a transient increase/decrease of bile flow under hypo- and hypertonic perfusion conditions, respectively. Exposure to hypotonic perfusion medium (185 mOsmol/l), the GSSG release into bile decreased significantly by 36% ( $P < 0.05$ ) and increased significantly by 34% ( $P < 0.05$ ) during exposure to hypertonic perfusion medium (345 mOsmol/l). The GSSG release into the perfusate was not detectable both during iso- and anisotonic exposures. The effect of anisotonicity on biliary GSSG excretion persisted throughout the anisotonic exposure and reversed back to control levels upon restoration of normotonic perfusion conditions (figure 2).

**Table 1.** Effects of anisotonicity on bile flow, GSSG release into bile, water content, and  $^{14}\text{CO}_2$  and LDH efflux from the perfused liver of *C. batrachus*.

Condition	Bile flow ( $\mu\text{l/g}$ liver/min)	GSSG release (nmol/g liver/min)	$1\text{-}^{14}\text{CO}_2$ efflux (nmol/g liver/min)	$6\text{-}^{14}\text{CO}_2$ efflux (nmol/g liver/min)	LDH efflux (mU/g liver/min)	Water content (%)
Normotonic	$1.05 \pm 0.07$	$1.18 \pm 0.09$	$18.4 \pm 1.52$	$7.55 \pm 0.75$	$78 \pm 6.7$	$76.5 \pm 2.5$
Hypotonic	$1.11 \pm 0.15$ (+ 5.7)	$0.75 \pm 0.06$ (- 36)	$38.72 \pm 3.14$ (+ 110)	$8.14 \pm 0.81$ (+ 8)	$69 \pm 5.5$ (- 11.5)	$84.0 \pm 2.7$ (+ 9.8)
	$P$ NS	$P$ < 0.05	$P$ < 0.001	$P$ NS	$P$ NS	$P$ NS
Hypertonic	$0.99 \pm 0.07$ (- 5.7%)	$1.58 \pm 0.11$ (+ 34)	$12.08 \pm 1.21$ (- 34)	$7.10 \pm 0.87$ (- 6)	$85 \pm 8.5$ (+ 9)	$68.5 \pm 1.8$ (- 10.4)
	$P$ NS	$P$ < 0.05	$P$ < 0.05	$P$ NS	$P$ NS	$P$ NS
Glutamine + glycine (2 mM each)	$1.09 \pm 0.08$ (+ 3.8)	$0.78 \pm 0.08$ (- 34)	$31.32 \pm 3.32$ (+ 70)	$7.97 \pm 0.88$ (+ 6)	$72 \pm 6.3$ (- 7.7)	$82.4 \pm 2.9$ (+ 7.7)
	$P$ NS	$P$ < 0.05	$P$ < 0.05	$P$ NS	$P$ NS	$P$ NS

Individual values of each parameter was taken only when it reached the steady state level during perfusion and was expressed as mean  $\pm$  SEM ( $n = 3\text{--}6$ ).

Percentage increase (+)/decrease (-) of parameters during anisotonic exposures compared to respective normotonic controls are given in parentheses.

NS, not significant.

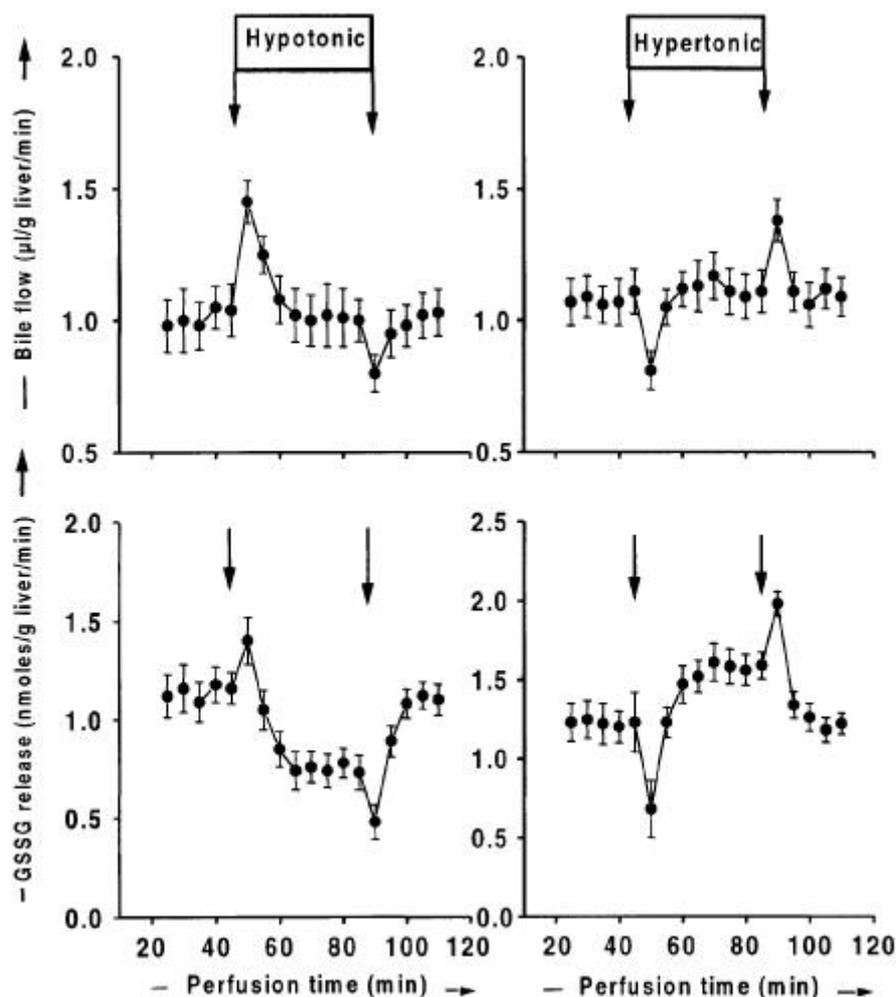
### 3.2 Effect of glutamine and glycine on GSSG release into bile and $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ glucose and $[6-^{14}\text{C}]$ glucose

Addition of glutamine plus glycine (2 mmol/l each) into the perfusate caused increase of hydration status (7.7%) resulting to a swelling of hepatocytes in the perfused liver of *C. batrachus*, similar to the situation as observed under hypotonic perfusion conditions (table 1). Glutamine plus glycine-induced cell swelling also affected the steady-state GSSG release into bile, thus causing a significant decrease of GSSG release into bile by 34% ( $P < 0.05$ ), but without affecting the steady state flow of bile (table 1). Addition of glutamine plus glycine in the perfusate also stimulated the production of  $^{14}\text{CO}_2$  from  $[1-^{14}\text{C}]$ glucose significantly by 70% ( $P < 0.01$ ), but without affecting the  $^{14}\text{CO}_2$  production from  $[6-^{14}\text{C}]$ glucose (table 1).

Both during iso- and anisotonic exposures, and also in the presence of glutamine plus glycine in the perfusate only negligible amount of LDH efflux was observed in the perfusate ranging between 70 to 85 mU/g liver/min (table 1).

### 3.3 Effects of anisotonicity on GSSG release into bile, $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ glucose in the presence of *t*-BOOH and *t*-BOOH-induced cell injury

As such the GSSG release into bile was stimulated by the infusion of *t*-BOOH (50  $\mu\text{mol/l}$ ) from  $1.18 \pm 0.003$  to  $3.97 \pm 0.24$  nmol/g liver/min (236%) under normotonic perfusion conditions (table 2). The steady state efflux of GSSG release into bile in the presence of *t*-BOOH (50  $\mu\text{mol/l}$ ) was also affected by anisotonicity, thus causing a 24% decrease ( $P < 0.05$ ) during hypotonic and 29% increase ( $P < 0.05$ ) of GSSG release during hypertonic exposures



**Figure 2.** Effects of anisotonicity on bile flow and biliary GSSG release from the perfused liver of *C. batrachus*. Values are plotted as mean  $\pm$  SEM ( $n = 3$ ).

(table 2). The glutamine plus glycine-induced cell swelling also caused a significant decrease of GSSG release into bile by 20% ( $P < 0.05$ ).

t-BOOH infusion itself caused slight but significant increase of  $^{14}\text{CO}_2$  from  $[1-^{14}\text{C}]$ glucose (35%,  $P < 0.05$ ), which further increased by 108% and 89%, respectively, during hypotonic exposure and during infusion of glutamine plus glycine together; hypertonic exposure caused 40% decrease of  $^{14}\text{CO}_2$  produced from  $[1-^{14}\text{C}]$ glucose (table 2), similar to the observations made in the absence of t-BOOH (compare figure 1; table 1).

Infusion of t-BOOH (50  $\mu\text{mol/l}$ ) as such caused significant increase of LDH efflux (416%,  $P < 0.001$ ) from the perfused liver under normotonic perfusion conditions, compared to the liver perfused without t-BOOH, which was further stimulated by 97% due to hypertonic cell shrinkage; hypotonic and glutamine plus glycine-induced cell swelling caused significant inhibition of LDH efflux by 61 and 58%, respectively (table 2).

In another set of experiment, t-BOOH was infused into the perfused liver at a concentration of 0.5 mmol/l 30 min after onset of iso-, hypo- and hypertonic exposures to assess the relative rates of hydroperoxide-induced cell injury under iso- and anisotonic conditions. After 90 min of infusion of t-BOOH, the LDH efflux in the perfusate was recorded to be  $787 \pm 55$  mU/g liver/min during normotonic exposure, which significantly decreased and increased to  $378 \pm 35$  and  $1680 \pm 90$  mU/g liver/min during hypotonic and hypertonic exposures, respectively (figure 3).

#### 4. Discussion

Perfusion of *C. batrachus* liver with hypotonic medium ( $-80$  mOsmol/l) caused 9.8% increase, and with hyper-

tonic medium ( $+80$  mOsmol/l) caused 10.4% decrease of water content in the perfused liver (table 1), similar to the observations made earlier (Goswami and Saha 1998). Although these changes of water content under anisotonic conditions were not significant compared to normotonic perfusion, yet were sufficient enough to cause significant changes of some metabolic fluxes as reported in the present study and also earlier by Goswami and Saha (1998). Likewise, addition of glutamine plus glycine (2 mmol/l each) in the influent also caused 7.7% increase of water content thus causing a cell swelling in the perfused liver (table 1), similar to the situation reported in rat liver (Häussinger and Lang 1991).

Normally during metabolism of  $[1-^{14}\text{C}]$ glucose, labelled  $\text{CO}_2$  is liberated by decarboxylation reaction in the pentose-phosphate pathway, as well as in the citric acid cycle (Nelson and Cox 2000). In the latter case,  $^{14}\text{CO}_2$  is produced from  $^{14}\text{C}$ -labelled pyruvate derived from glycolysis, which in our experiment was markedly diluted by the addition of unlabelled lactate (1.2 mmol/l) and pyruvate (0.3 mmol/l) in order to minimize  $^{14}\text{CO}_2$  production via the citric acid cycle or utilization of  $^{14}\text{C}$ -labelled pyruvate for other reaction. In addition, the contribution of the citric acid cycle to  $\text{CO}_2$  was also estimated from  $^{14}\text{CO}_2$  production from  $[6-^{14}\text{C}]$ glucose, which will not pick up  $\text{CO}_2$  via the pentose-phosphate pathway. Hypotonic exposure did not cause any change of  $^{14}\text{CO}_2$  production from  $[6-^{14}\text{C}]$ glucose, but markedly stimulated  $^{14}\text{CO}_2$  production from  $[1-^{14}\text{C}]$ glucose (110%) (figure 1; table 1). These findings suggest a stimulation of flux through pentose-phosphate pathway following hypotonic cell swelling. Conversely, hypertonic cell shrinkage reduced  $^{14}\text{CO}_2$  production from  $[1-^{14}\text{C}]$ glucose (34%), but had no effect on  $^{14}\text{CO}_2$  produc-

**Table 2.** Effects of anisotonicity on bile flow, GSSG release into bile,  $^{14}\text{CO}_2$  and LDH efflux in the presence of t-butylhydroperoxide (50  $\mu\text{mol/l}$ ) from the perfused liver of *C. batrachus*.

Condition	Bile flow ( $\mu\text{l/g}$ liver/min)	GSSG release (nmol/g liver/min)	$1-^{14}\text{CO}_2$ efflux (nmol/g liver/min)	LDH (mU/g liver/min)
Normotonic	$1.22 \pm 0.11$	$3.97 \pm 0.56$	$24.15 \pm 3.86$	$395 \pm 35$
Hypotonic	$1.34 \pm 0.12$ (+ 9.8)	$3.02 \pm 0.33$ (- 24)	$50.32 \pm 5.22$ (+ 108)	$151 \pm 22$ (- 61)
	<i>P</i> NS	$< 0.05$	$< 0.001$	$< 0.01$
Hypertonic	$1.14 \pm 0.09$ (- 6.5)	$5.14 \pm 0.49$ (+ 29)	$17.37 \pm 1.17$ (- 40)	$778 \pm 59$ (+ 97)
	<i>P</i> NS	$< 0.05$	$< 0.01$	$< 0.001$
Glutamine + glycine (2 mM each)	$1.28 \pm 0.08$ (+ 4.9)	$3.17 \pm 0.37$ (- 20)	$45.66 \pm 6.91$ (+ 89)	$164 \pm 27$ (- 58)
	<i>P</i> NS	$< 0.05$	$< 0.01$	$< 0.01$

Individual values of each parameter were taken only when it reached the steady state level during perfusion and were expressed as mean  $\pm$  SEM ( $n = 3-6$ ).

Percentage increase (+)/decrease (-) of parameters during anisotonic exposures compared to respective normotonic controls are given in parentheses.

NS, not significant.

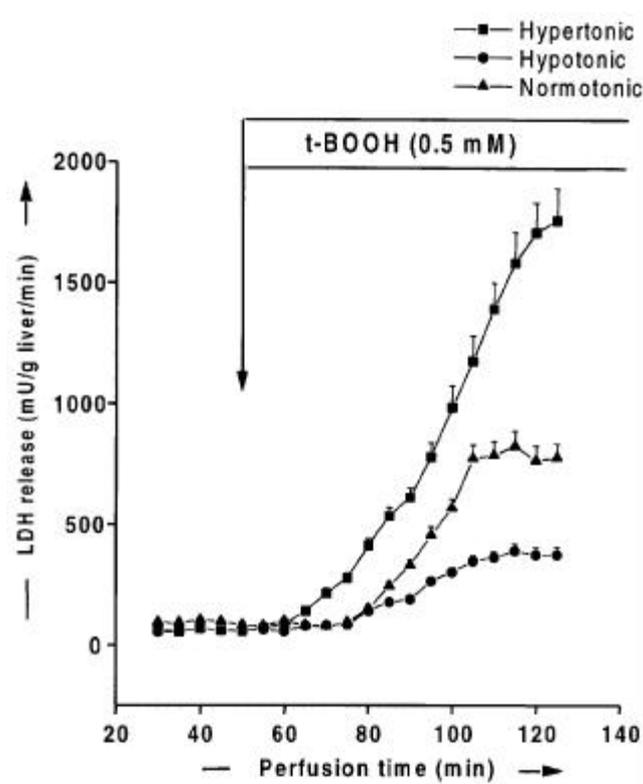
tion from [6-<sup>14</sup>C]glucose, pointing to a diminished flux through pentose-phosphate pathway following hypertonic cell shrinkage. Amino acid-induced cell swelling also caused an increase in the flux of <sup>14</sup>CO<sub>2</sub> production (70%) from [1-<sup>14</sup>C]glucose, suggesting that the cell volume changes is the main driving force to these metabolic responses. Further, the effect of anisotonicity on <sup>14</sup>CO<sub>2</sub> production from [1-<sup>14</sup>C]glucose also persisted in the presence of t-BOOH (50 μmol/l) although t-BOOH itself increased slightly but significantly the <sup>14</sup>CO<sub>2</sub> production (table 2). These effects of anisotonicity may add to other recently described effects of cell volume on hepatic carbohydrate metabolism, such as inhibition of glycogenolysis and gluconeogenesis and stimulation of glycogen synthesis during cell swelling, and vice versa during cell shrinkage (Goswami and Saha 1998; C Goswami, S Datta, K Biswas and N Saha, unpublished results).

Stimulation of flux through pentose-phosphate pathway by hypotonic- and amino acid-induced cell swelling would also stimulate NADPH production, which could be utilized for stimulatory activity of conversion of GSSG to GSH. Therefore, the inhibition of GSSG efflux observed during cell swelling due to hypotonicity as well as during amino acid-induced cell swelling and an increase of GSSG efflux during cell shrinkage, observed in the present study (figure 2; table 1), could be correlated with the activity of pentose-phosphate pathway.

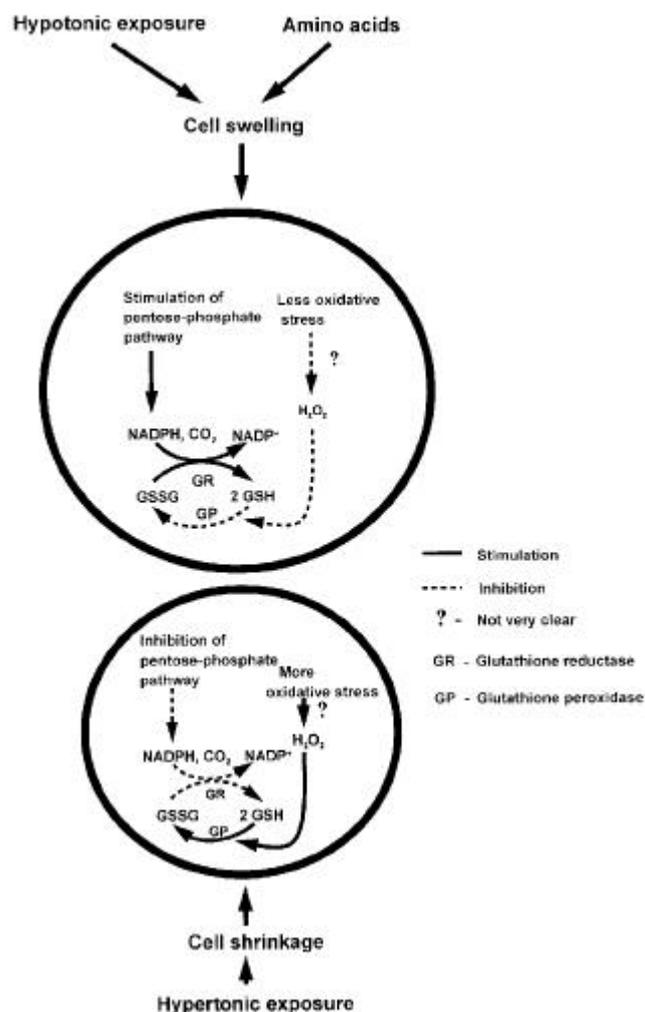
The inhibitory and stimulatory effects of GSSG release into bile by hypotonic cell swelling and hypertonic cell shrinkage, respectively, can not be explained due to changes of bile flow, since the bile flow by the perfused liver remained same during anisotonic exposure except for a transient increase or decrease of bile flow during hypotonic and hypertonic exposures, respectively (figure 2; table 1). Further, the changes of GSSG release in the bile during anisotonic exposure may not be due to alterations of paracellular permeability as indicated by a lack of anisotonic effects on the rapid appearance of bolus-injected horseradish peroxidase in bile shown at least in rat liver (Häussinger *et al* 1992). Similar effects on GSSG release into bile were observed even when t-BOOH was present at a concentration of 50 μmol/l (table 2). The inhibitory effect of hypotonicity on GSSG release may be related to increase of hydration status or cell swelling, since amino acid-induced cell swelling also decreased GSSG release into bile (table 1). Increase of GSSG release into bile during hypertonic exposure is suggestive that hypertonic cell shrinkage is accompanied by an increased net GSH oxidation and loss, not only during addition of t-BOOH but also in its absence. GSSG release into bile was suggested to be linearly related to the hepatocellular GSSG content in rat liver (Akerboom *et al* 1982; Sies 1985), and also was suggested as an indicator for oxidative stress (Sies 1985). Therefore, the present observations could

point to a modulation of cellular thiol status by anisotonicity. It is not very clear with the present results whether anisotonic exposure affects the endogenous formation of reactive oxygen species or whether it affects protective mechanisms such as flux through glutathione reductase. With respect to the latter, a swelling-induced stimulation of the pentose-phosphate pathway should favour NADPH formation for glutathione reductase. An increased regeneration of GSH from GSSG could then explain the swelling-induced decrease in GSSG release into bile as observed in the present study (figure 1; table 1).

Sustained oxidative stress is known to impair cell viability, frequently assessed as LDH leakage from the cells. Both under iso- and anisotonic conditions the LDH release from the perfused liver was very negligible averaging to about 75 mU/g liver/min. However, infusion of t-BOOH (50 μmol/l), which is known to cause oxidative stress (Brigelius 1983; Sies 1985), significantly stimulated the LDH release into the effluent even under isotonic conditions. This was further stimulated under hypertonic cell shrinkage and inhibited during hypotonic cell swelling (table 2). Similar observations were also made when t-BOOH at a concentration of 0.5 mmol/l was infused for 90 min while



**Figure 3.** Effects of anisotonicity on LDH release in the effluent in the presence of t-butylhydroperoxide (t-BOOH; 0.5 mmol/l) from the perfused liver of *C. batrachus*. Values are plotted as mean  $\pm$  SEM ( $n = 3$ ).



**Figure 4.** Hypothetical model to explain the effects of cell volume changes on the oxidative stress-related metabolic pathways in the hepatocyte of *C. batrachus*.

perfusing with iso- and anisotonic media with a more pronounced effect of anisotonicity on LDH release (figure 3). One of the reasons of cell injury leading to changes of LDH release could be due to changes of GSH level in the hepatocytes, since hypertonicity caused more release of GSSG into bile. Normally GSH is known as an important substrate for enzymatic antioxidant function and also is capable of non-enzymatic radical scavenging (Sies 1985). An increase in the flux of GSH from the perfused rat liver into the effluent under hypotonic perfusion condition was reported earlier (Häussinger *et al* 1990). It may be suggested that t-BOOH under hypertonic condition causes more oxidative stress, possibly following lipid peroxidation and cell rupture, thereby resulting in an increase of LDH release in the effluent. Hypotonicity on the other hand results in a reverse situation. Saha *et al* (1992) suggested that the hypotonically swollen hepatocytes are less

susceptible to hydroperoxide induced membrane damage, and is related to different degrees of membrane stretching and multiple effects of anisotonicity on the cytoskeleton and hepatocellular metabolism. Considerably less leakage of t-BOOH-induced LDH from hypotonically swollen cells than from hypertonically shrunken cells, as observed in the present study, is suggestive that cell swelling may exert beneficial effects during early stages of oxidative cell injury probably due to swelling-induced alterations in hepatic metabolism (figure 4).

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