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METABOLIC AND ENZYMATIC HETEROGENEITY IN THE LIVER OF THE UREOGENIC TELEOST *OPSANUS BETA*

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Summary

Viable toadfish hepatocytes were separated into distinct subpopulations by gradient centrifugation. Although 3–5 density subpopulations were obtained for each fish, only two metabolically and enzymatically different subpopulations could be discerned. In all cases, hepatocytes with the lowest density (less than $1.040\,g\,ml^{-1}$) were more oxidative in scope, as judged by the activities of mitochondrial enzymes (citrate synthase, aspartate aminotransferase, glutamate dehydrogenase); activities of these enzymes (normalised to cell protein) were on average two- to threefold higher than in subpopulations with higher densities. Lower-density hepatocytes also contained higher levels of the urea cycle enzymes arginase and ornithine carbamoyltransferase. The higher-density subpopulations showed no significant differences from each other in enzymatic activities. Compared with lower-density cells, these hepatocytes had higher activities of two cytosolic enzymes, malate dehydrogenase and glutathione-*S*-transferase. There was no distinct distribution pattern for alanine aminotransferase and glutamine synthetase. Despite generally lower oxidative enzyme content, higher-density hepatocytes were metabolically more active, with 2.5- to fourfold higher rates of urea synthesis, gluconeogenesis and oxidation of lactate. We conclude that, although the toadfish liver shows distinct enzymatic and metabolic heterogeneity, this heterogeneity is dissimilar to the zonation pattern in the livers of mammals, in that separated toadfish hepatocyte types did not appear to possess exclusive metabolic functions. Notably, all cells were capable of metabolic functions that are strictly localised in mammalian liver. In nitrogen metabolism, glutamine synthetase displays a distribution pattern commensurate with its unique metabolic function in the liver of the ureogenic toadfish. Further, all subpopulations possessed detoxification capabilities as indicated by high levels of glutathione-*S*-transferase, a 'phase II' conjugation enzyme.

Key words: hepatocytes, toadfish, ureogenesis, gluconeogenesis, enzymes, bicarbonate, density gradient centrifugation, glutamine synthetase, *Opsanus beta*.

Introduction

The mammalian liver reveals distinct functional zones, mainly composed of perivenous and periportal hepatocytes (Jungermann and Katz, 1989), which can be distinguished by different experimental approaches (Katz, 1989). One of the metabolic pathways exhibiting pronounced zonation is the ornithine–urea cycle and closely linked enzymes such as alanine aminotransferase (Shank *et al.* 1959), glutaminase and glutamate dehydrogenase (Lamers *et al.* 1988). Urea synthesis, as judged by direct assessment from amino acids other than glutamine (Pösö *et al.* 1986) or by carbamoylphosphate synthetase distribution (CPS I; Gaasbeek Janzen *et al.* 1984, 1987), prevails in the periportal zone, i.e. 'upstream' in the hepatic circulation. Glutamine synthetase, which appears to have assumed the function of preventing hepatic efflux of unmetabolised ammonia (Stoll and Häussinger, 1989), occurs only in a small number of perivenous ('downstream') hepatocytes surrounding the hepatic venule (Gebhardt and Mecke, 1983; Gaasbeek Janzen *et al.* 1987).

Most teleosts are ammoniotelic and only very few species rely on the energetically costly *de novo* synthesis of urea as a route for removing excess 'nitrogen' in a non-toxic form. Regardless of the ultimate role of urea, these ureogenic fishes (with the exception of the lungfishes) use an identical metabolic pathway for their hepatic urea synthesis (Mommsen and Walsh, 1989). Ureogenic species possess a glutamine-dependent carbamoylphosphate synthetase (CPS III; Anderson, 1976, 1980), instead of the ammonia-dependent carbamoyl phosphate synthetase I of mammals, in conjunction with a mitochondrial rather than a cytosolic arginase (Mommsen and Walsh, 1989). Since fish utilise ammonia rather than glutamine as their main vehicle for nitrogen transport, glutamine synthetase becomes the 'nitrogen' entry point for hepatic urea synthesis, which is in contrast to its 'clean-up' role in mammals.

Heterogeneity and some zonation, albeit with enzyme groupings distinct from those in mammals, can also be detected in the teleostean liver (Schär *et al.* 1985; Mommsen *et al.* 1991; Ottolenghi *et al.* 1991), suggesting that functional heterogeneity of liver may be an early trait in vertebrate evolution. Therefore, it appeared that toadfish, belonging to the small group of constantly ureogenic teleosts, would present an ideal model for studying evolutionary trends in metabolic heterogeneity in general and urea synthesis in particular.

The three prevailing approaches to the study of metabolic heterogeneity of liver have been immunocytochemistry, the isolation of different populations of hepatocytes by gradient centrifugation and the selective destruction of the unwanted species of liver cell by digitonin (reviewed by Katz, 1989). While some laboratories are focusing on the enzymatic heterogeneity of fish liver using histochemical techniques (Schär *et al.* 1985; Sastry *et al.* 1976; Hampton *et al.* 1988), experiments with digitonin have yielded somewhat ambiguous results, possibly because of the differing hepatic microcirculation in fishes (Ottolenghi *et al.* 1991; Mommsen *et al.* 1991). We have previously used gradient centrifugation (Mommsen *et al.* 1991) to identify subpopulations of differing density in trout (*Oncorhynchus mykiss*) live

following isolation of viable hepatocytes by conventional techniques (Moon *et al.* 1985). Although this specific technique fails to furnish information about spatial relationships between cell types and may, at least in the rat, potentially decrease the cellular responsiveness to insulin (Memon *et al.* 1989), it is well suited for analysing the existence of hepatic heterogeneity. Here we use gradient centrifugation to extend our initial observations of metabolic heterogeneity of the teleostean liver to the marine toadfish and focus particularly on the heterogeneity of the urea synthetic pathway and selected associated enzymes.

Materials and methods

Animals

Gulf toadfish (*Opsanus beta*, 35–125 g) were obtained from a local Miami fisherman in November 1989 and maintained in the laboratory at ambient temperature (26°C). Animals were kept without feeding for a maximum of 1 week.

Hepatocytes

Fish were anaesthetised with neutralised MS222 ($0.5\,g\,l^{-1}$). Liver cells were isolated by *in situ* perfusion with Hanks' medium adapted for toadfish (TH), containing 0.03 % collagenase (Walsh, 1987). Hepatocyte isolation, initial purification and separation by gradient centrifugation were made in TH. For metabolic studies, cells obtained after gradient centrifugation were diluted with modified TH medium (THB), adjusted to give final concentrations of 2 % defatted bovine serum albumin and $1.0\,mmol\,l^{-1}$ $CaCl_2$. Suspensions containing 50–100 mg cell ml^{-1} were stored for up to 4 h on ice until experimentation. No changes in individual subpopulation patterns were observed during storage.

Gradient centrifugation

Samples (0.75–2.0 ml) of the final cell suspension were layered on top of a discontinuous density gradient. The gradient consisted of five layers (4.5 ml each) of Percoll ($1.130\,g\,ml^{-1}$) diluted with TH to give 20, 32.4, 37.8, 41.6 and 54.0 % Percoll. Cells were separated by centrifugation at room temperature (RT=22°C) in a swing-out rotor for 10 min at 130 ***g***, followed by 5 min at 540 ***g***. Separated cell subpopulations were recovered at or slightly above an interface. Density calibration was done with a set of coloured marker beads (Sigma Chemical Company, St Louis, MO, USA).

Subpopulations of cells were sampled from the interfaces by suction (a maximum volume of 1.6 ml was removed) and transferred into microcentrifuge tubes. For the assessment of metabolic activities, a sample (up to 800 μl) of these cell suspensions was diluted with THB to give a maximum Percoll concentration of 17 % and used directly. A 10 μl sample was removed for immediate protein determination (Bradford, 1976). The remainder of the cell suspension was diluted with a small volume of TH and centrifuged at 13 000 ***g*** for 2 min, as was a sample of

the freshly isolated cells. The resulting pellets were quickly frozen in liquid nitrogen. Samples were stored at −76°C for up to 4 days.

Metabolic activities

Rates of urea synthesis, lactate gluconeogenesis and lactate oxidation were measured in freshly isolated hepatocytes or hepatocyte subpopulations using ^{14}C radiotracer techniques (urea synthesis, Mommsen and Walsh, 1989; gluconeogenesis and oxidation, Walsh, 1989). Experiments were conducted in duplicates in a volume of 1.0 ml for 90 min at RT and terminated by the addition of 100 μl of 70 % perchloric acid. Original cell isolates and subpopulations were assayed concurrently. A preliminary experiment with freshly isolated cells established that Percoll concentrations of 10, 13, 15 or 17 % did not alter metabolic flux through the above pathways.

Enzymes

Frozen cell pellets were taken up in 200 or 400 μl of ice-cold sonication medium (1 mmol l^{-1} dithiothreitol, 0.5 mmol l^{-1} EDTA and 20 mmol l^{-1} potassium phosphate in 50 % glycerol, adjusted to pH 7.5) and sonicated on ice three times for 5 s each at full power. Samples were centrifuged for 30 s at 13 000 *g* and the supernatants were used within 60 min. The following nine enzymes were measured: malate dehydrogenase (MDH, EC 1.1.1.37), alanine and aspartate aminotransferases (AAT, EC 2.6.1.2, DAT, EC 2.6.1.1, respectively), glutamate dehydrogenase (GDH, EC 1.4.1.3), citrate synthase (CS, EC 4.1.3.7), glutathione-*S*-transferase (GST, EC 2.5.1.18), glutamine synthetase (GS, EC 3.6.1.2), ornithine carbamoyltransferase (OCT, EC 2.1.3.3) and arginase (ARG, EC 3.5.3.1). Enzymatic activities were assessed in duplicates either by direct spectrophotometry (AAT, DAT, GDH, CS, MDH, GST) or by colorimetric determination of product after a timed interval (OCT, ARG, GS). Assays were done at room temperature under saturation conditions as outlined elsewhere (Habig *et al.* 1974; Mommsen and Walsh, 1989; Mommsen *et al.* 1991). Prior to assays, we confirmed that small amounts of Percoll (5 %) did not alter enzymatic activities. Activities of lactate, glucose-6-phosphate and isocitrate (NADP) dehydrogenases, phosphoenolpyruvate carboxykinase, pyruvate kinase and fructose 1,6-bisphosphatase were below 5 units g^{-1} fresh mass and were thus considered to be too slow to be measured under the time constraints of our experiments. Enzyme activities are given in milliunits (nmol mg^{-1} h^{-1}) per milligram of sample protein.

Biochemicals

Percoll was obtained from Pharmacia, USA. Radiotracers ($NaH^{14}CO_3$, specific activity 7 Ci mol^{-1}, and [U-^{14}C]lactate, specific activity 126 Ci mol^{-1}) were supplied by New England Nuclear. Coupling enzymes came from Boehringer Mannheim, Lachine, PQ. Other biochemicals were purchased from Sigma Chemical Company, St Louis, MO, USA.

Statistical analysis

Statistical analysis was done by analysis of variance (ANOVA), followed by Newman–Keuls test, using a statistical package (SYSTAT). In some cases, values were log-transformed to meet the assumptions of ANOVA. In one case, Kruskal–Wallis analysis was required because data were not homoscedastic (Sokal and Rohlf, 1981). 5 % was used as the fiducial level of significance.

Results

Isolated toadfish hepatocytes are an ideal system in which to analyse aspects of liver metabolism in a ureogenic teleost. The cell preparation is metabolically highly competent, viable for at least 9 h without deterioration (stable ATP content, unchanged rates of urea and glucose synthesis), contains less than 2 % of cells that take up Trypan Blue, and contamination with non-parenchymal cells is less than 5 %. Using the discontinuous gradient described in Table 1, freshly isolated toadfish hepatocytes can be further separated into 3–5 subpopulations with different densities. Although one subpopulation tends to predominate in individual separations, accounting for about half of all cells applied to the gradient, a more homogeneous distribution of protein over the gradient is noticed when averaged over 10 preparations (Table 1). Nevertheless, the subpopulation with the highest density generally contains one-third of the protein separated and also has the lowest rank number of all subpopulations (Table 1). After centrifugation, all cells are fully viable, as shown by their continued exclusion of Trypan Blue, metabolic activity and response to hormones (T. P. Mommsen and P. J. Walsh, unpublished results). In addition, contamination with red blood cells is nonexistent. Toadfish red blood cells are of higher density than any of the hepatocytes and

Table 1. *Relative protein concentration in five different subpopulations of toadfish hepatocytes*

	Density ($g\,ml^{-1}$)	Protein (% of total recovered)	Rank*
Subpopulation 1	<1.040	12.1±1.5	3.9±0.3
Subpopulation 2	<1.055	22.1±6.5	3.4±0.4
Subpopulation 3	<1.062	13.7±3.5	3.6±0.5
Subpopulation 4	<1.066	19.5±2.8	2.1±0.3
Subpopulation 5	<1.081	33.1±5.7	2.0±0.3

Densities were calculated from the dilutions of Percoll and confirmed with density marker beads.

Values are presented as means±S.E.M. of duplicate determinations made separately on hepatocytes isolated from 10 fish.

*Subpopulations were assigned ranks according to their relative abundance in the individual preparations (rank 1=most abundant; rank 5=least abundant).

consequently sediment to the bottom of the centrifuge tubes. Although we did not detect any differences in cell size between subpopulations, the amount of internal granulation, visible at 40× magnification, appeared to increase with increasing cell density.

Enzymatic activities

Two superimposed patterns can be discerned when the different subpopulations are analysed for enzymatic activities. The lightest cell subpopulation (density below $1.040\,g\,ml^{-1}$) is characterised by considerably higher activities of citrate synthase, aspartate aminotransferase and glutamate dehydrogenase, whereas malate dehydrogenase and glutathione-*S*-transferase predominate in higher-density subpopulations (Table 2). Two enzymes associated with the production of urea in the toadfish liver, namely ornithine carbamoyltransferase and arginase, are more prevalent in the lowest-density subpopulation (Table 2). Results on the heterogeneous distribution of these enzymes are highly significant (Table 3). Although, at first glance, glutamine synthetase appears to have the same distribution as citrate synthase, this trend is statistically not significant (Table 3) and, thus, the enzyme can be grouped with alanine aminotransferase as lacking heterogeneous distribution among subpopulations.

It is possible that the distribution patterns observed merely reflect the abundance of mitochondria in the respective subpopulation, since all enzyme activities predominating in the lower-density fraction belong to mitochondrial enzymes. To test this hypothesis, all enzyme activities were normalised to the activity of citrate synthase, an acknowledged marker for the inner mitochondrial membrane. Following this transformation, the subpopulation-specific differences for glutamate dehydrogenase and ornithine carbamoyltransferase disappear (Table 4A), whereas differences for two other mainly mitochondrial enzymes in toadfish, aspartate aminotransferase and arginase, are still significant. *Vice versa*, when activities are normalised to the activity of malate dehydrogenase, a predominantly cytosolic enzyme in the toadfish liver, subpopulation-specific differences for glutathione-*S*-transferase vanish (Table 4B). None of these treatments changes the results of the statistical analysis for alanine aminotransferase or glutamine synthetase, supporting the above conclusions.

As the statistical analysis (Table 3) of the enzyme data (Table 2) clearly shows, only two patterns in enzyme distribution between the different subpopulations can be discerned. Of all nine enzymes, only one case is found where a significant difference in activity exists *among* the four higher-density subpopulations. In this case, subpopulation 2 contains significantly less malate dehydrogenase activity than subpopulation 5. Therefore, it seems acceptable to combine the four denser fractions and treat them as one subpopulation with identical enzymatic behaviour, distinctly different from that of the lightest hepatocytes.

Metabolic activities

For the measurement of metabolic activities a much larger sample was required,

Table 2. *Enzyme activities in subpopulations of toadfish hepatocytes*

	Enzyme								
	CS^{L}	MDH^{H}	AAT^{NS}	DAT^{L}	GDH^{L}	GS^{NS}	GST^{H}	OCT^{L}	ARG^{L}
Cell mixture before centrifugation									
	78±8	3555±250	1672±156	1964±147	1014±119	131±18	7192±655	2774±238	344±29
Subpopulations after gradient centrifugation									
Subpopulation 1	120±13	2454±161	1345±157	2273±229	1570±232	184±27	3894±504	4172±377	401±38
Subpopulation 2	41±5	2721±265	1360±132	1410±147	516±32	173±89	5341±640	1624±125	204±17
Subpopulation 3	32±5	3194±309	1450±188	1247±135	523±104	85±10	6289±979	1351±157	162±18
Subpopulation 4	38±5	3725±304	1609±205	1531±138	499±82	97±19	7406±1078	1427±193	150±14
Subpopulation 5	42±6	3698±278	1605±179	1595±180	503±77	98±19	5939±670	1365±161	139±23

Activities are given in nmol min^{-1} mg^{-1} protein±S.E.M. for 10 independent determinations at 22°C under saturation conditions.

CS, citrate synthase; MDH, malate dehydrogenase; AAT, alanine aminotransferase; DAT, aspartate aminotransferase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GST, glutathione-*S*-transferase; OCT, ornithine carbamoyltransferase; ARG, arginase.

For detailed statistical analyses, see Tables 3 and 4.

^{L} Subpopulations with lower densities contain significantly more enzyme activity than higher-density subpopulations.

^{H} Subpopulations with higher densities contain significantly more enzyme activity than the lighter cells.

^{NS} No significant differences in enzyme activity between subpopulations.

Table 3. *Statistical treatment of enzyme activities in subpopulations of toadfish hepatocytes from Table 2*

	Enzyme								
	CS†	MDH	AAT	DAT	GDH†	GS†	GST	OCT†	ARG
Significance	S	S	NS	S	S	NS	S	S	S
Cell mixture	1	1		3	1–5		1	1–5	2–5
Subpopulation 1	*	4, 5		*	*		4	*	*
Subpopulation 2	–	5		–	–		–	–	–
Subpopulation 3	–	–		–	–		–	–	–
Subpopulation 4	–	–		–	–		–	–	–

S, significant ($P<0.05$); NS, not significant; numbers indicate the subpopulation(s) from which the enzyme activity in this particular subpopulation differs significantly.

* Significantly different from all other subpopulations.

In cases where the enzyme data were not homoscedastic (i.e. $P<0.02$, in a Bartlett's test, indicated by †), data were log-transformed to meet the assumptions of ANOVA.

Abbreviations for enzymes are given in Table 2.

than for the enzyme assays. However, we still wanted to conduct our analysis on individual livers rather than on pooled material. Therefore, we were forced to select those two or three subpopulations that were most prominent following gradient centrifugation and ignore the others. In all six cases, we sampled the lightest subpopulation, which collected on top of 20 % Percoll, and one or two subpopulations with higher densities. The denser subpopulations show no significant differences in metabolic performance and data are therefore combined. In contrast to the abundance of mitochondrial enzymes (see Table 2), higher-density cells are metabolically more active than the lowest-density subpopulation of hepatocytes (Table 5). This statement holds true for urea synthesis, lactate oxidation and lactate gluconeogenesis, with ratios ranging from 2.6 to 3.8 (Table 5). Corresponding ratios for enzymatic activities range from around 1–2 for alanine aminotransferase, malate dehydrogenase and glutathione-*S*-transferase, to 0.5–0.3 for all the others.

Discussion

The toadfish liver contains metabolically and enzymatically distinct subpopulations of hepatocytes which can easily be separated by density gradient centrifugation. The results of our study therefore extend the previous observations for rainbow trout on hepatocyte heterogeneity (Mommsen *et al.* 1991) to another teleost. Although toadfish hepatocytes can be separated into a minimum of three metabolically competent subpopulations, only two enzymatic patterns can be discerned: lighter cells with higher levels of oxidative enzymes and denser hepatocytes characterised by higher rates of urea and glucose synthesis and lactate oxidation as well as by higher activities of two cytosolic enzymes. As in the

Table 4A. *Statistical treatment of enzyme activities in subpopulations of toadfish hepatocytes normalised to the activity of citrate synthase*

	Enzyme							
	MDH	AAT	DAT	GDH	GS	GST	OCT	ARG
Significance	S	S	S	NS	NS	S	NS	S
Subpopulation 1	3–5	*	*			3, 4		3
Subpopulation 2	–	–	–			–		–
Subpopulation 3	–	–	–			–		5
Subpopulation 4	–	–	–			–		–

Table 4B. *Statistical treatment of enzyme activities in subpopulations of toadfish hepatocytes normalised to the activity of malate dehydrogenase*

	Enzyme							
	CS	AAT	DAT	GDH	GS	GST	OCT	ARG
Significance	S	NS	S	S	NS	NS	S	S
Subpopulation 1	3–5		*	*			*	*
Subpopulation 2	–		–	–			3–5	3–5
Subpopulation 3	–		–	–			–	–
Subpopulation 4	–		–	–			–	5

S, significant ($P<0.05$); NS, not significant; numbers indicate the subpopulation(s) from which the enzyme activity in this particular subpopulation differs significantly.

* Significantly different from all other subpopulations.

All ratios were log-transformed to meet the assumptions of ANOVA.

In the case of OCT, normalised to CS, data were not homoscedastic, even after log-transformation. Therefore, a non-parametric test was used (Kruskal–Wallis).

Abbreviations for enzymes are given in Table 2.

rainbow trout, the enzymatic pattern differs substantially from that in mammalian liver, where oxidative enzymes prevail in one subpopulation (periportal cells), while less oxygen-dependent pathways and phase II detoxification are distinctive properties of another subpopulation (perivenous cells).

Although the exact physiological role of urea synthesis in the toadfish is not clear (Mommsen and Walsh, 1989; Walsh *et al.* 1990), the isolated liver cells synthesize large amounts of urea from glutamine and bicarbonate, followed by release of urea into the medium. In mammals, where ammonia is the substrate for carbamoylphosphate synthetase I, cells with urea synthetic activities are spatially and functionally separated from liver cells containing glutamine synthetase. The latter enzyme is localised to prevent ammonia efflux from the liver into the bloodstream. No such differentiation is apparent in the toadfish liver. In this fish, *all* hepatocyte subpopulations recovered from the gradient possessed urea cycle activity, with lighter cells displaying lower activity, in spite of the fact that the lighter cells contained higher titres of some of the urea cycle enzymes. Glutamine

Table 5. *Metabolic activities in subpopulations of toadfish hepatocytes*

	Urea synthesis	Glucose synthesis	CO_2 production
Lower-density cells	16.3±4.8	21.0±6.6	15.1±5.6
Higher-density cells	45.2±15.7	43.4±18.9	45.0±6.1
Ratio (high density/low density)	3.8±0.9	2.6±0.8	3.5±0.8

Metabolic activity is expressed as nanomoles of product per milligram of protein per hour.
Values are given as means±S.E.M. of six independent determinations in duplicates.
Metabolic rates of the freshly isolated hepatocytes, related to fresh masses of cells, are (N=6) 4.04±1.74 μmol g^{-1} h^{-1} of urea synthesized from bicarbonate, 3.19±0.99 μmol g^{-1} h^{-1} of glucose synthesized from lactate and 5.62±1.23 μmol g^{-1} h^{-1} of carbon dioxide released from lactate.
Ratios for glucose and carbon dioxide are given for five observations; one pair of observations was removed from the analysis as an outlier – actual rates were well outside four standard deviations of the new mean.

synthetase, the enzyme providing carbamoylphosphate synthetase III with one of its substrates, was distributed homogeneously among the different subpopulations of toadfish liver cells. Thus, its distribution agrees with its function as the feeder enzyme for urea synthesis and clearly shows that the toadfish liver enzyme occupies a position where it can detoxify ammonia only in conjunction with urea synthesis. In the mammalian liver, glutaminase is instrumental in delivering 'nitrogen' to the urea cycle, while glutamine synthetase merely functions to prevent spillover of ammonia from perivenous hepatocytes into the hepatic venous circulation. Further, it is clear that the distribution patterns of carbamoylphosphate synthetase and glutamine synthetase, together with the specific function of glutamine synthetase in the toadfish, preclude the reciprocal control of these two enzymes, as occurs in the rat liver (de Groot *et al.* 1987).

We therefore conclude that, although metabolic and enzymatic heterogeneity can be demonstrated in the toadfish liver, the enzyme pattern is quite distinct from that familiar from mammalian systems but similar to that found in trout liver (Mommsen *et al.* 1991). There are three main differences between toadfish and rat liver: (1) the distribution of alanine aminotransferase and glutamine synthetase is homogeneous in the toadfish liver; (2) in the toadfish, as in the rainbow trout, glutamate dehydrogenase distributes together with citrate synthase; and (3) urea synthesis in the toadfish liver is an asset not restricted to specialised cells.

Although the experimental procedure was adequate to separate subpopulations of toadfish hepatocytes by density alone, other techniques will have to be utilised to supply information about the exact spatial relationships of hepatocytes within the toadfish liver. One possibility deserving further study is that hepatocytes from different regions of the fish liver are themselves heterogeneous with regard to density. Although previous histochemical studies (Schär *et al.* 1985; Sastry *et al.* 1976; Hampton *et al.* 1988) demonstrate the unlikeliness of this situation, in the

extreme case, our specific experimental approach would yield a randomised distribution of function, where, in fact, spatial heterogeneity may exist.

At present we can only speculate why those cells with higher titres of enzymes involved in urea synthesis actually display lower rates of urea synthesis than subpopulations with smaller amounts of such enzymes as ornithine carbamoyltransferase, arginase or aspartate aminotransferase. Our data suggest that these enzymes do not limit the rate of urea synthesis and thus it does not come as a surprise that enzyme titres do not reflect the rates of urea synthesis. Although glutamine synthetase can be induced by treatment of toadfish with a glucocorticosteroid (T. P. Mommsen, E. Danulat and P. J. Walsh, in preparation), our results also indicate that glutamine synthetase is not alone in determining urea synthetic rates in the toadfish liver. Other likely candidates in this role are carbamoylphosphate synthetase III and the enzymatic steps leading to and from argininosuccinate. Experiments have been initiated to analyse these hypotheses further.

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