CONJUGATION OF ORGANIC COMPOUNDS IN ISOLATED HEPATOCYTES FROM A MARINE FISH, THE PLAICE, *PLEURONECTES PLATESSA*

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Abstract—A method is described for the preparation of viable hepatocytes from a marine fish, the plaice. Their ability to detoxify organic compounds was measured by the formation of glucuronic acid and sulphate conjugates with the model substrates 1-naphthol and phenolphthalein. 1-Naphthol was conjugated three- to four-fold faster than phenolphthalein and glucuronidation predominated with both substrates. Strong substrate inhibition of glucuronidation was observed with 200 μM 1-naphthol or phenolphthalein. No measurable sulphate conjugation was detected with phenolphthalein. Treatment of fish with 3-methylcholanthrene induced formation of both glucuronide and sulphate conjugates by two- to three-fold. Compared with rat hepatocytes, the extent of sulphation was 100-fold lower in plaice hepatocytes whereas glucuronide formation was only 10-fold lower. The observations indicate that isolated plaice hepatocytes provide a suitable system for studies of the detoxication of xenobiotic pollutants in fish liver.

Many lipophilic compounds, both exogenous and endogenous, are metabolized by the liver to form water-soluble conjugates before excretion from the body. Despite earlier claims to the contrary, fish are capable of producing glucuronide and sulphate conjugates [1, 2], although this is an area which has received little attention and the relative importance of these reactions in fish is unknown. In most mammals, glucuronidation is quantitatively the most important reaction although many compounds conjugated with glucuronic acid are also conjugated with sulphate, these two conjugation reactions often competing for the same compound. The relative contribution of each route is determined by several factors, including the structure and lipophilicity of the substrate [3, 4], differences in the kinetics of the enzymes involved [5, 6], the availability of cofactors [7] and differential induction by externally administered agents [8, 9].

Some characteristics of UDP-glucuronyltransferase (EC 2.4.1.17) activity have been described in microsomal fractions from the livers of several fish using bilirubin [10, 11] and 4-nitrophenol [12, 13] as substrates. However, the enzymes have not been studied in depth and the relative contributions of glucuronidation and sulphation to the detoxication and elimination of pollutant xenobiotic compounds

in fish remains to be defined. In relation to the latter, it should be noted that extrapolation of data obtained *in vitro*, mainly from subcellular hepatic fractions, to the situation *in vivo* is extremely complicated. The enzymes have different subcellular localizations, with glucuronyltransferases being bound to the microsomal membrane [13], and sulphotransferases (EC 2.8.2) being cytosolic [14]. The latency of glucuronyltransferase in the microsomal membrane and its modulation by processes causing membrane perturbation [13] and by endogenous effectors [13, 15] add a further complication.

Extensive use has been made of isolated rat hepatocytes to study the integration of drug metabolism [16] and a comparable system for fish would be extremely useful for studies of the detoxication of environmental pollutants. In the present study we have developed a procedure for the isolation of viable hepatocytes from a demersal marine flatfish, the plaice, *Pleuronectes platessa*. This fish ingests xenobiotics accumulated by benthic organisms at lower trophic levels and is a species used for standard toxicity testing in the United Kingdom. Despite this, data for xenobiotic metabolism in the species are lacking.

We describe here the use of a plaice hepatocyte preparation to study the conjugation of the model compounds, 1-naphthol and phenolphthalein. In rats, 1-naphthol is a substrate for a 3-methylcholanthrene-inducible form of glucuronyltransferase [17, 18] the activity of which develops in the late foetal cluster [19]. Phenolphthalein glucuronyltransferase activity is induced by phenobarbitone [18] and develops in the rat neonate [19]. Alterations in the pattern of conjugation with increasing substrate concentration, and the effect of treatment with 3-methylcholanthrene (3-MC)‡ were investigated using these substrates.

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‡ Abbreviations used: 1-NA, 1-naphthol; 1-NG, 1-naphthol glucuronide; 1-NS, 1-naphthol sulphate; 3-MC, 3-methylcholanthrene; HEPES, N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid.

MATERIALS AND METHODS

(i) Fish

Sexually immature plaice weighing 250–350 g were acclimated in a flowing sea-water aquarium for 7–14 days. Some fish were injected i.p. with 10 mg/kg 3-MC (1% in olive oil) and sacrificed 3 days after injection, when induction of cytochrome P-450 and transferase activities in this species is maximal [20].

(ii) Preparation of hepatocytes

The procedure was modified from Moldeus et al. [21] for rat liver and Walton and Cowey [22] for trout liver. It was impossible to obtain satisfactory perfusion of the liver either via the hepatic artery or by using multiple cannulation of the vessels of the hepatic portal system. Consequently retrograde perfusion via the bile duct was used to prepare the hepatocytes.

Three solutions were used, all equilibrated with 95% O_2/5% CO_2. Solution A was a modified Hank's medium (NaCl 8.0 g, KCl 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $Na_2HPO_4 \cdot 2H_2O$ 0.06 g, KH_2PO_4 0.06 g and $NaHCO_3$ 2.1 g in 1 l.) containing 0.6 mM ethanedioxybis(ethylamine)tetracetate and 10 mM HEPES, pH 7.4. Solution B was the same Hank's medium containing 4 mM $CaCl_2$, 10 mM HEPES and 1 mg/ml collagenase. For Solution C the Hank's medium was supplemented with 1% defatted bovine serum albumin, 2.5 mM $CaCl_2$ and 10 mm HEPES. Perfusions were carried out at room temperature (21–23°).

Fish were anaesthetized with MS222 Sandoz in sea-water (1:2000 w/v) and injected via the caudal vein with 1000 μg/kg heparin (in 0.9% NaCl) to prevent blood clotting during operative procedures. After 10 min recovery in sea-water, they were anaesthetized again and the wall of the abdominal cavity removed. The bile duct was ligated at its point of entry into the intestine and then the duct was punctured just below the gall bladder with a 21 guage needle and a 1.02 mm diameter polythene cannula inserted about 0.5 cm towards the intestine and held in position with an artery clip and ligature. Perfusion with solution A was commenced at 1.2 ml/min. The hepatic artery and hepatic portal veins were severed to prevent build up of back pressure. The liver and its cannulated bile duct were then dissected free from the alimentary canal, surrounding mesentery and spleen and transferred to a perfusion table. After 5–10 min open perfusion, recirculation of perfusate containing collagenase (solution B) was commenced. After 30–45 min perfusion with this solution, all extrahepatic tissue was removed and the liver cells were dispersed by gentle stirring into solution C. The suspension was filtered through nylon gauze (0.5 mm mesh) and the cells were sedimented at 500 g for 10 min. The cells were resuspended in Krebs–Henseleit buffer (NaCl 8.04 g, KCl 0.412 g, KH_2PO_4 0.187 g, $MgSO_4 \cdot 7H_2O$ 0.342 g, $CaCl_2 \cdot H_2O$ 0.658 g and $NaHCO_3$ 2.2 g in 1 l.) containing 10 mM HEPES, pH 7.4, gassed with O_2/CO_2 and kept on ice until use.

(iii) Determination of viability

Viability was routinely assessed by Trypan blue exclusion [21] and cell concentration determined in an improved Neubauer haemocytometer. Sedimented cells were fixed for electron microscopy in 2.5% glutaraldehyde, 0.5% NaCl and 0.1 M phosphate buffer (pH 7.2, 650 mOsm), dehydrated in graded alcohols and embedded in Spurr resin. Ultra-thin sections were stained in uranyl acetate/lead citrate and examined in a JEOL 100CX scanning transmission electron microscope operated in the transmission mode.

(iv) Incubations

Incubations were performed within 1 hr of cell isolation at 23° in round-bottomed 50-ml flasks under 95% O_2/5% CO as described by Hogberg and Kristoferson [23] with 0.8–4.7 × 10^6 cells/ml in Krebs–Henseleit buffer containing 10 mM HEPES, pH 7.4. 1-Naphthol was added in acetone to a final concentration in the incubation mixture of 0.2% v/v acetone and phenolphthalein in methanol to a final concentration of 0.2% v/v methanol. These solvent concentrations do not affect the transferase activities in rat hepatocytes [24].

In one set of incubations 2 × 10^6 cells were incubated in 5 ml Krebs–Henseleit buffer containing 10 mM HEPES, pH 7.4 in 20-ml flasks on a Gilson differential respirometer. CO_2 was absorbed by placing 200 μl 15% KOH in the centre well of each flask with a "wick" of filter-paper to increase the area for absorption. Incubations were carried out for 135 min at 21° with gentle shaking and O_2 consumption was recorded every 15 min.

(v) Analysis of conjugates

Samples of the hepatocyte incubation mixtures (1 ml) were taken at intervals, 1 ml methanol was added and, after standing on ice for 30 min, the precipitated proteins were pelleted by centrifugation at 4000 g for 15 min. The samples were stored at −15° until analysed.

Conjugates were analysed by reversed-phase ion-pair high pressure liquid chromatography (HPLC) as described previously [25, 26]. The stationary phase was a Spherisorb ODS 2 column, 250 × 5 mm with 5 μm packing (Anachem Ltd., Luton, U.K.) and the mobile phase was 60% methanol/40% water containing 20 mM tetrabutylammonium hydrogen sulphate. The compounds were detected by u.v. absorbance at 220 nm for 1-naphthol and 230 nm for phenolphthalein and quantitated by peak height measurements using appropriate standards. For all compounds measured the standard curves were linear over the range of concentrations used.

Chemicals. Substrate compounds, their glucuronide and sulphate derivatives, HEPES, MS 222 and tetrabutylammonium hydrogen sulphate were obtained from Sigma Chemical Co., Poole, U.K.; collagenase was obtained from Boehringer Mannheim Ltd., Lewes, U.K.; methanol (HPLC grade) was obtained from Fisons Ltd., Loughborough, U.K. All other chemicals were of the highest purity available from BDH Ltd., Poole, U.K.

RESULTS

Cell preparation and viability

One small lobe at the top of the liver had a bile duct passing directly into the neck of the gall bladder and the cannulation procedure did not permit perfusion of this region. The remainder of the organ was completely digested after collagenase perfusion. Cell yield was routinely about $150–200 \times 10^6$/g liver, a slightly higher value than that obtained for rat liver [21]. The cells appeared well rounded in the light microscope although there was some tendency to aggregate. Viability, assessed by Trypan blue exclusion, was $97 \pm 2\%$ (N = 7). Electron microscopy showed that most cells were separate, well rounded with intact cell membranes. The endoplasmic reticulum and mitochondria were not swollen and the mitochondrial cristae well defined. The cells were typical of teleost liver, containing many lipid droplets and glycogen deposits. Some cells were still attached by junctional complexes to adjacent cells, thus explaining the aggregates of two to four cells observed in the light microscope.

During incubations with 1-naphthol and phenolphthalein the viability was monitored by Trypan blue exclusion every 30 min and no significant decline in viability was detected after 2 hr exposure to 200 μM 1-naphthol or 100 μM phenolphthalein (viability remained greater than 94% with both substrates).

Oxygen consumption during incubation of cells with 200 μM 1-naphthol and 100 μM phenolphthalein was also measured as an indicator of viability. The results are shown in Fig. 1. Rates of oxygen consumption were linear for the first 90 min of incubation, declining slightly thereafter. Oxygen consumption in the substrate-containing flasks was only slightly lower than in controls. Cell viability after 140 min assessed by Trypan blue exclusion, was 89% for control, 95% for 100 μM phenolphthalein and 93% for 200 μM 1-naphthol.

Conjugation of 1-naphthol

Incubations were carried out for 2 hr at four concentrations of 1-naphthol and samples were taken at intervals for analysis of conjugates. Production of 1-naphthol glucuronide over 60 min was proportional to cell concentration at all substrate concentrations (Fig. 2). The time courses for glucuronide formation by hepatocytes from control fish with 10–200 μM 1-naphthol in the incubation mixtures are shown in Fig. 3. For the first hour of incubation the rates were linear with time for concentrations of 1-naphthol up to 50 μM. After 60 min the substrate concentrations were appreciably lower (or entirely consumed) and consequently the rates of conjugation were lower. The hatched curve for 20 μM 1-naphthol shows an incubation with a low cell concentration where the substrate was only 36% consumed; in this case reaction linearity was maintained.

With 200 μM 1-naphthol, glucuronide formation was severely restricted—2.0 nmoles 1-naphthol glucuronide/10^6 cells was formed after 120 min compared with 7.5 nmoles with 50 μM substrate. A similar phenomenon was not observed with rat hepatocytes (Table 3), although Mulder and Pilson [27] have reported substrate inhibition of phenolphthalein glucuronidation in rat liver microsomes. The optimum substrate concentration for glucuronide conjugation by plaice hepatocytes was in the region of 50 μM 1-naphthol (Fig. 4).

Formation of 1-naphthol sulphate was also proportional to the concentration of hepatocytes in the incubations, although the rate of formation decreased markedly with time (Fig. 3). There was very little increase in sulphate conjugation with 1-naphthol concentrations greater than 10 μM (Fig. 4). The inhibition of glucuronidation at 200 μM 1-naphthol was not observed with sulphate formation, indicating that the former was due to enzyme inhibition rather than cellular toxicity. Sulphation of 1-naphthol was between 5 and 10% of glucuronidation at all substrate concentrations used (Fig. 4). Increasing the inorganic sulphate concentration in the incubation medium has been reported to increase sulphate conjugation of 4-methylumbelliferone in rat hepatocytes [8]. When we increased the sulphate concentration from the standard 0.8 mM to 8 mM in incubations with 20 μM 1-naphthol, we observed a small but non-significant difference in the amount of
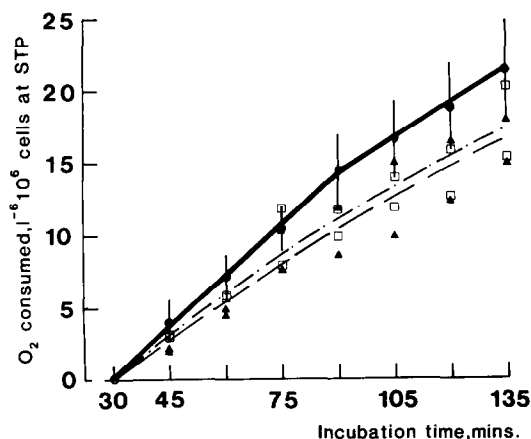

Fig. 1. Oxygen uptake by isolated plaice hepatocytes. Hepatocytes were incubated with shaking in respirometer flasks at 21° and O_2 consumption measured after 30 min equilibration. ●———● Controls, no added substrate, mean $\pm$ S.D., N = 5; □—·—□ 100 μM phenolphthalein, two determinations; ▲———▲ 200 μM 1-naphthol, two determinations.
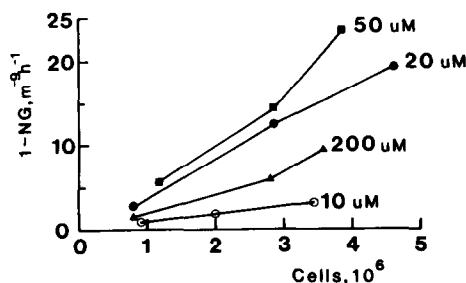

Fig. 2. Formation of 1-naphthol glucuronide (1-NG) by different concentrations of isolated plaice hepatocytes. Hepatocytes were incubated for 1 hr at 23° and the glucuronide formation was measured by reversed-phase ion-pair HPLC.
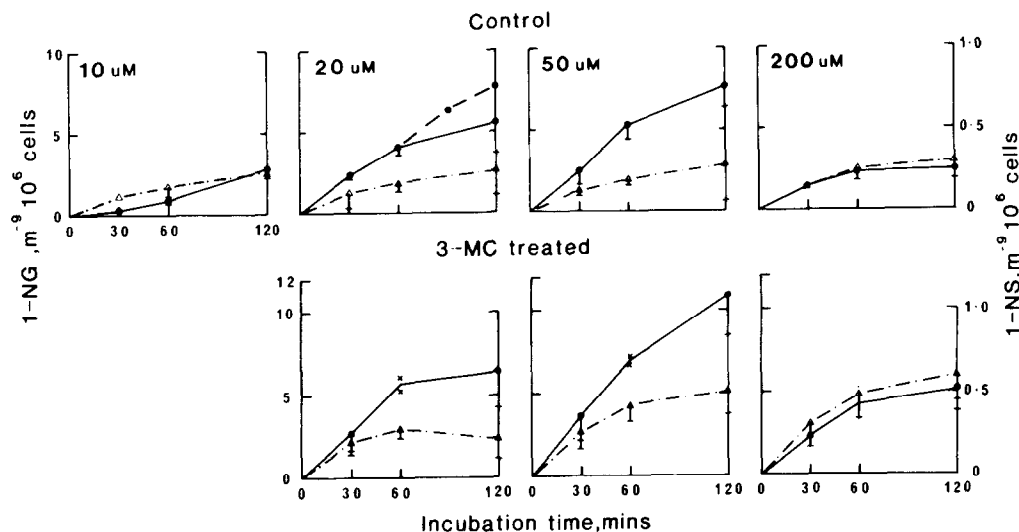
Fig. 3. Time course of conjugation of 1-naphthol by isolated plaice hepatocytes from control and 3-MC-treated fish. Isolated hepatocytes were incubated at 23° with varying initial concentrations of 1-naphthol. The conjugates formed were separated and quantitated by HPLC. Mean −S.D. for three cell preparations (*, individual values where $N = 2$). ●——● 1-Naphthol glucuronide (1-NG); ●——● 1-naphthol glucuronide where substrate concentration was not limiting; △·—·△ 1-naphthol sulphate (1-NS); note scale differences.

1-naphthol sulphate formed $(0.44 \pm 0.09$, cf. 0.3 ± 0.06 nmoles/10^6 cells/hr).

Treatment of fish with 3-MC increased both the glucuronidation and sulphation of 1-naphthol (Fig. 3 and Table 1). Glucuronidation was increased 1.5-fold and sulphation two-fold. The ratio of 1-naphthol glucuronide/sulphate formed was 24 in control fish and 17 in 3-MC-treated fish, indicating a slightly greater increase in sulphation compared with glucuronidation (Table 2).

Conjugation of phenolphthalein

When hepatocytes were incubated with phenolphthalein and the conjugates analysed by HPLC, glucuronidation, but not sulphation, was observed. At a substrate concentration of 20 μM, formation of phenolphthalein glucuronide was linear with time up to 2 hr and the rate of glucuronidation was about
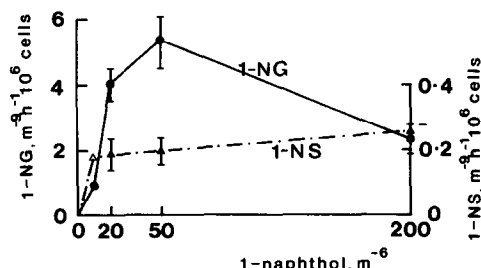

Fig. 4. Concentration dependence of the conjugation of 1-naphthol. Isolated hepatocytes from control fish were incubated for 1 hr at 23° with 1-naphthol and conjugates analysed by HPLC. Mean ± S.D. for three cell preparations. ●——● 1-Naphthol glucuronide (1-NG); △—·—△ 1-naphthol sulphate (1-NS); note scale differences.

one third that of 1-naphthol (Table 1). Treatment of the fish with 3-MC increased phenolphthalein glucuronidation to a greater extent than that of 1-naphthol (2.5-fold compared with 1.5-fold). At 200 μM phenolphthalein, very strong substrate inhibition was observed and glucuronide formation was not linear with time.

DISCUSSION

In this paper we have described a preparation of isolated plaice hepatocytes suitable for studying the detoxication of pollutant xenobiotic chemicals in fish liver. Hepatocytes from rats have been used extensively to investigate metabolism and toxicity of a wide range of xenobiotics. Due to difficulties inherent in the in vivo investigation of the integration of detoxication and excretion of xenobiotics in fish it is important to develop and establish this alternative method of assessing pollutant metabolism. Therefore the ability of plaice hepatocytes to conjugate model compounds was evaluated with reference to data which is available for rat hepatocytes.

Plaice hepatocytes conjugate 1-naphthol three- to four-fold faster than phenolphthalein (Table 1) and preliminary data have shown that 4-nitrophenol is conjugated more efficiently than 1-naphthol. Glucuronidation predominates over sulphation with both substrates at all concentrations tested (Fig. 3). In fact, no measurable sulphate conjugation was detected with phenolphthalein. Table 3 shows that rat hepatocytes also form 1-naphthol conjugates faster than those of phenolphthalein and that sulphation of phenolphthalein is very much lower than that of 1-naphthol [16]. The rate of conjugation by plaice hepatocytes (measured at 23°) is more than 10-fold lower than in rat hepatocytes (measured at

Table 1. Rates of glucuronide conjugation with different substrates in hepatocytes isolated from control and 3-MC-treated plaice*

Substrate	Initial concentration (μM)	Treatment	
		Control	3-MC
		(nmoles gluguronide formed/ 10^6 cells/hr)	
Phenolphthalein	20	1.56, 0.95 N = 2	3.17 ± 1.13 N = 3
	200	0.18, 0.1 N = 2	0.33, 0.33 N = 2
1-naphthol	10	0.94 ± 0.09 N = 3	—
	20	4.06 ± 0.43 N = 3	5.3, 6.02 N = 2
	50	5.33 ± 0.84 N = 3	7.25, 6.76 N = 2
	200	2.29 ± 0.46 N = 3	4.35 ± 1.39 N = 3

* Isolated hepatocytes were incubated with different substrates for 1 hr at 23° and the conjugates analysed. Results are given as means ± S.D. for different hepatocyte preparations.

37°). These experiments on rat hepatocytes were carried out using identical conditions to those reported in the present paper except the cells were incubated at 37° rather than 25°, the optima for the two species [1, 16].

The extent of sulphate formation with 1-naphthol in plaice is about 100-fold lower than that in the rat (Table 3). Sulphation does not increase appreciably at substrate concentrations above 10 μM and it is not limited by the concentration of inorganic sulphate in the buffer. This indicates that it is limited by the total activity of the sulphotransferase and that the reaction is saturated at 10 μM 1-naphthol. In rat hepatocytes, sulphation is saturated at 25 μM with 2-naphthol, phenolphthalein and 4-nitrophenol [16, 28]. A decrease in the extent of sulphation at increasing substrate concentrations has also been demonstrated to occur *in vivo* in rats [29] and it has been shown that the K_m of the sulphotransferase is much lower than that for the glucuronyltransferase [5, 6]. Conjugation of sulphate with benzo(a)pyrene metabolites, 2-naphthol and estrone has been reported in fish [2, 33] and plaice hepatocytes have demonstrable sulphotransferase activity, although this is 10–100-fold lower than that in rats [14, 30, 31]. A two-fold induction of 1-naphthol sulphation by 3-MC was

here observed in plaice hepatocytes. In comparison, inducers of drug metabolism, including 3-MC, polychlorinated biphenyls and phenobarbitone, do not induce sulphation in the rat [5, 6].

Little is known about the properties of the conjugating enzymes in fish. The K_m values for glucuronyltransferase activity with 4-nitrophenol have recently been reported for untreated "native" liver microsomes from rainbow trout [1] and are similar to reported values in untreated rat liver microsomes [30, 31]. After allowance for temperature differences (25° vs 37°) the rates of 4-nitrophenol glucuronidation in trout and rat microsomes were about the same. In a comparative study, the glucuronyltransferase activities with 4-nitrophenol of perch, roach and vendace were all found to be lower than those of trout [32]. The strong substrate inhibition of glucuronidation in plaice hepatocytes was also observed in trout microsomes at 4-nitrophenol concentrations greater than 100 μM [1]. Although substrate inhibition has been reported for mammalian glucuronyltransferase, especially with

Table 2. The ratios of glucuronide/sulphate formed with 1-naphthol by plaice hepatocytes in control and 3-MC-treated fish*

Substrate concentration (μM)	1-NG/1-NS	
	Control	3-MC-treated
10	6.7	—
20	21.4	18.9
50	26.7	16.3
200	9.2	8.9

* Data calculated during phase of linear metabolism from Fig. 3.

Table 3. Comparison of glucuronide formation in plaice and rat hepatocytes*

	Substrate concentration (μM)		Glucuronide	Sulphate
			(nmoles/10^6 cells/hr)	
Plaice				
	Phenolphthalein	20	1.26	n.d.
		200	0.14	n.d.
	1-Naphthol	20	4.06	0.19
		200	2.29	0.25
Rat				
	Phenolphthalein	25	16.8	4.2
		200	55.2	4.2
	1-Naphthol	20	44.4	30.6
		100	142.8	40.2

* Data for plaice hepatocytes taken from Fig. 3; for rat hepatocytes data for phenolphthalein taken from [16], data for 1-naphthol unpublished. n.d.—not detectable.

phenolphthalein [13, 27], it is not so marked as in fish and its significance in xenobiotic detoxication is worthy of further investigation. In a recent study, the scope of the glucuronidation reactions in trout liver with a number of endogenous and exogenous compounds was compared with that in several mammalian species [2]. Compared with activities in the rat, phenol glucuronyltransferase activity (4-nitrophenol and 1-naphthol) was very much lower, bilirubin glucuronyltransferase was about half, whilst 17-hydroxysteroid glucuronyltransferase activity (testosterone) was about five-fold greater in trout. The differences in the range of glucuronyltransferase activities between fish and mammals may be an indication of variations in the proportions of different isoenzymes in the livers; in plaice liver microsomes we have observed induction of phenol glucuronyltransferase by 3-MC [20] and this is confirmed in the present study with hepatocytes.

It appears that there are clear and important differences in the conjugative metabolism of xenobiotic compounds in fish and mammals arising from differences in enzyme activities. This emphasizes the importance of defining metabolic transformations of pollutant xenobiotics and the effects of these compounds on the hepatic enzymes of detoxication in fish. Isolated hepatocytes can be a valuable tool for such investigations.

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