Table 2. Effect of praseodymium (3 mg/kg i.v.) on the amounts of cytochromes P-450 and b_5 in rat liver smooth (SER) and rough (RER) endoplasmic reticulum and whole microsomes 24 and 48 h after injection

	P-450 (nmoles/mg protein)	Amount (%)	b_5 (nmoles/mg protein)	Amount (%)
Microsomes				
Control	1.02±0.13*	100.0	0.76±0.11	100.0
24 h	0.80±0.12	78.4	0.56±0.10	73.7
48 h	0.58±0.13	56.8	0.44±0.12	57.9
SER				
Control	1.11±0.15	100.0	0.76±0.16	100.0
24 h	1.02±0.16	91.9	0.62±0.12	81.6
48 h	1.18±0.10	106.3	0.70±0.08	92.1
RER				
Control	1.11±0.16	100.0	0.73±0.15	100.0
24 h	0.52±0.12	46.9	0.34±0.16	46.6
48 h	0.26±0.12	23.4	0.26±0.12	35.6

* ±SD.

the level of both cytochrome P-450 and b_5 and the activities of drug metabolizing enzymes such as AHH and AH in rat liver. A similar trend was also seen in these enzyme activities in both SER and RER subfractions (table 1). When compared to their corresponding controls the enzyme activities in the RER were more affected. This can be due to the different distribution pattern of light lanthanons between these subfractions. When another light lanthanon, cerium, was injected as Ce-144 i.v. into male rats, nearly all the radioactivity in liver microsomes was found in the RER subfraction (unpublished observation).

The fate of the cytochromes P-450 and 5_5 is shown in table 2. Interestingly, no significant decrease in the cytochrome level was observed in the SER subfraction whereas in the RER a marked decrease was seen. The distinct behaviour of the enzyme activities and the changes in both cytochrome levels may partly be due to changes in the phospholipid content of these fractions after Pr-treatment[9]. Pr was found to increase the phospholipid content in the SER but to decrease the phosphatidylcholine content in the RER. Because the microsomal electron transport chain is known to be composed of 3 main components[10]: a flavoenzyme, phosphatidylcholine and cytochrome P-450 the lack of one of these components, phosphatidylcholine, may be the main reason for the pronounced impairment of drug metabolism especially in the RER subfraction.

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Inhibition of uridinediphosphate glucuronyltransferase caused by furosemide

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Summary. The diuretic agent, furosemide, inhibits liver microsomal uridinediphosphate glucuronyltransferase (EC. 2.4.1.17), in monkey and rat. Inhibition is of the non-competitive type.

Furosemide (4-chloro-N-furfuryl-5-sulfamoylanthranilic acid) is a widely used saluretic in the treatment of edema and hypertension. Many attempts have been made in order to clarify its metabolic fate in the body. Furosemide is mainly eliminated by the kidneys where it is secreted by the proximal tubule cells[3]. Recent investigations[4–9] have revealed that furosemide and its metabolites are excreted via the bile route, too. We could demonstrate that one of the metabolites of the diuretic is the glucuronide[10].

The present paper is concerned with the question whether furosemide has an inhibitory effect on microsomal uridinediphosphate glucuronyltransferase (EC. 2.4.1.17) of liver microsomes in vitro, as this is the enzyme which is responsible for the formation of furosemide glucuronide.

Materials. The following chemicals were used in this study: Ammonium acetate, acetylacetone, guaiacol, salicylic acid, p-nitrophenol, saccharose (from Merck AG, Darmstadt), 4-methylumbelliferone (from EGA-Chemie, Steinheim bei Heidenheim/Brenz), aminopyrine, acetylsalicylic acid, 2-hydroxy-2-ethylbutyryl-N,N-diethylamide (HOE 17,879), furosemide (from Hoechst AG, Frankfurt/Main), calcium chloride, acetic acid, trichloroacetic acid (from Riedel-De Haën, Seelze bei Hannover), and glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, nicotinamide adenine dinucleotide phosphate, uridinediphosphate glucuronic acid (from Boehringer, Mannheim).

Preparation of microsomes. Microsomes were prepared from rat (Wistar) and guinea-pig livers by the calcium chloride precipitation method[11–13] and from rhesus monkey livers by differential centrifugation at 10,000 and 100,000×g. The microsomal pellets obtained by these methods were suspended in isotonic potassium chloride solution in such a way that 1.0 ml of the suspension was equivalent to 100 mg of liver fresh weight.

Interaction of some compounds with glucuronyltransferase

Species	Substance	Enzyme activity (%)	Significance
Rhesus monkey	Control	100.0± 2.8	
	Furosemide, 10 mM	2.6± 1.8	$p<0.0005$
	Furosemide, 3 mM	41.0 *	*
	Morphine, 1 mM	84.9± 4.1	$p<0.0005$
	Guaiacol, 3 mM	19.9± 1.1	$p<0.0005$
	Salicylic acid, 3 mM	111.0± 3.1	n. sign.
	Probenecid, 10 mM	9.2± 1.0	$p<0.0005$
Rat	Control	100.0± 4.8	
	Furosemide, 10 mM	4.0 *	*
	Furosemide, 3 mM	40.0 *	*
	HOE 17,879, 10 mM	91.7± 6.7	n. sign.
	Probenecid, 1 mM	62.9± 13.2	$p<0.005$
	p-Nitrophenol, 1 mM	6.8± 1.2	$p<0.0005$

Values are percentage to control incubations (water instead of the inhibitor substance). Mean values ± SD; * only 1 determination was made (no significance calculated); p from t-test.

Enzyme assays. Uridinediphosphate glucuronyltransferase was assayed with 4-methylumbelliferone (7-hydroxy-4-methylcoumarin) as the substrate[14-18], in the absence and presence of the inhibitor compounds, in the following way: The reaction mixture was composed of 0.27 mM 4-methylumbelliferone, 30 mM magnesium chloride, 1.667 mM uridinediphosphate glucuronic acid (as the sodium salt), 166.7 mM tris(hydroxymethyl)aminomethane hydrochloride buffer pH 8.0, and microsomal suspension equivalent to 50 mg liver fresh weight, in a final volume of 3.0 ml. After starting the enzyme reaction by addition of uridinediphosphate glucuronic acid, the samples were incubated at 37 °C for 30 min, and then rapidly mixed with 6.0 ml of ice-cold 0.2 M glycine buffer pH 10.8. After centrifugation, fluorescence of the supernatant was read at an excitation wavelength of 385 nm and an emission wavelength of 445 nm, in a Perkin-Elmer fluorescence spectrophotometer. The reference in which uridinediphosphate glucuronic acid had been omitted was used as a standard. It was not necessary to include zero time values.

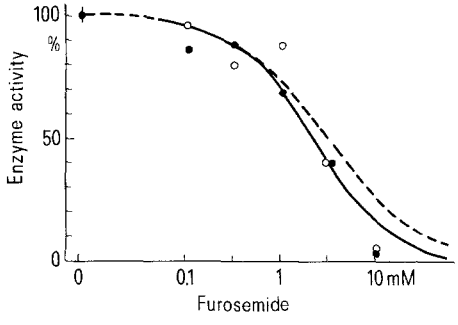

Fig. 1. Inhibition of microsomal uridinediphosphate glucuronyltransferase by furosemide. —●—, Rhesus monkey; —○—, rat.

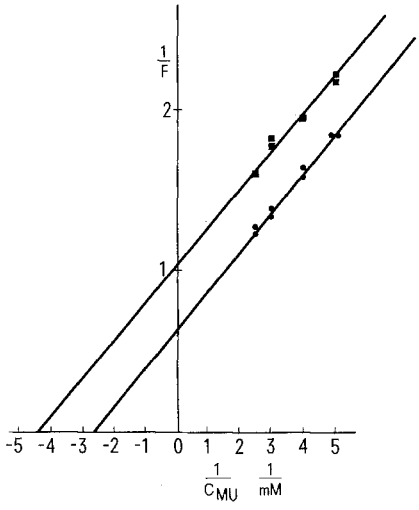

Fig. 2. Lineweaver-Burk plot of uridinediphosphate glucuronyltransferase with monkey microsomes. ■, Without furosemide; ●, with furosemide. Abscissa: 1/(concentration of 4-methylumbelliferone) (1/mM), ordinate: $1/\Delta F$.

Aminopyrine N-demethylase was measured by the method of Leber et al.[19], in the presence and absence of furosemide, in 12,000 × g supernatants prepared from rat liver homogenates in isotonic potassium chloride solution[20].

Results. Furosemide inhibits glucuronyltransferase activity, both in rat and monkey liver microsomes (figure 1 and table). Under the experimental conditions of the enzyme assay, 50% inhibition occurs at 2.2 mM in the rat, and at 3.2 mM in the monkey. As shown with monkey microsomes, the inhibition of uridinediphosphate glucuronyltransferase caused by furosemide is of the non-competitive type (figure 2). Probenecid also inhibits microsomal uridinediphosphate glucuronyltransferase, both in monkey and guinea-pig microsomes (figure 3) and in rat microsomes (table). Glucuronyltransferase, with 4-methylumbelliferone as the substrate, is inhibited by morphine, guaiacol,

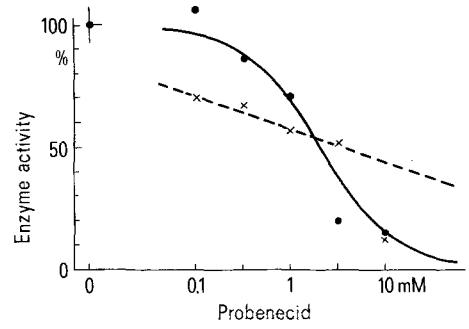

Fig. 3. Inhibition of microsomal uridinediphosphate glucuronyltransferase by probenecid. —●—, Rhesus monkey; ---×---, guinea-pig.

and nitrophenol, too, but not by salicylic acid and the compound HOE 17,879 (table). Aminopyrine N-demethylase which is inhibited by the compound HOE 17,879[21] is not inhibited by furosemide, in concentrations up to 10 mM.

Discussion. The fact that furosemide inhibits microsomal uridinediphosphate glucuronyltransferase, is to be seen in connection with the fact that it is glucuronidized by this enzyme itself[10]. So one would expect a competitive type of inhibition, but in fact the inhibition was shown to be a non-competitive one. The authors are reluctant to draw too many conclusions from this unexpected finding, because the type of inhibition needs to be studied with solubilized and purified enzyme preparations, rather than in crude microsomes, to investigate in detail the kind of interaction between furosemide and the conjugating enzyme. In contrast to the microsomal enzyme inducer, HOE 17,879 (2-hydroxy-2-ethylbutyryl-N,N-diethylamide) which inhibits drug-metabolizing, cytochrome P-450-dependent mixed-function oxidases of liver microsomes, both in vivo[20] and in vitro[21] but has no effect on glucuronyltransferase (see Beyhl[21], cf. table), furosemide does not influence aminopyrine N-demethylase in vitro which agrees well with the in vivo findings of Muschaweck and Beyhl (unpublished) namely that aminopyrine N-demethylase activity is not affected by furosemide, neither in acute nor in chronic treatment with this drug. From our studies with furosemide and some other diuretics as well as with probenecid, we have got the impression that the carboxyl group must be present for inhibition of the glucuronyltransferase, in this series of compounds.

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Autoradiographic evidence for binding of ^{3}H-flunitrazepam (Rohypnol®) to melanin granules in the cat eye

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Summary. This autoradiographic study revealed after an i.v. injection an accumulation of ^{3}H-flunitrazepam in melanin granules of the pigment epithelium and of the choroid cells. It seems that after a short period, a large proportion of the strongly bound drug is released.

Recently it was demonstrated by whole-body autoradiography (Prof. J. Rieder and Dr R. Heintz, personal communication) that flunitrazepam (Rohypnol®) shows an affinity to melanin-containing tissues, like the retinotoxic drug chloroquine[2-4]. After a single injection in pregnant mice, ^{14}C-marked flunitrazepam was visible in the uvea of pigmented offspring even 100 days after the injection. The present study examined the localization of ^{3}H-flunitrazepam in the tissues of the cat eye at an ultrastructural level, in connection with a toxicological study[5] of chloroquine and flunitrazepam.

^{3}H-flunitrazepam (labeled at position 9; sp. act. = 60.67 mCi/mg)[6] was used at a concentration of 2 mg/ml in the solvent as for human injection. 2 male cats of approximately 2.5 kg received 1 mg/kg via the saphenous vein in 20 min with an infusion pump. For this application the cats were anaesthetized with an intramuscular injection of 14 mg/kg Vetalar®. 2 other male cats were treated from Monday to Friday the 1st week and from Monday to Thursday the 2nd week with 1 mg/kg (total 9 injections) ^{3}H-flunitrazepam daily. The drug was injected into the left jugular vein of non-anaesthetized cats through a mounted Vercath catheter within 1–2 min each. After 1 and 14 days, respectively, the eyes were carefully dissected from narcotized cats (35 mg/kg Nembutal, i.p.), and cleaned of adherent tissue. An incision into the front of the cornea was made with a scalpel, and the eye fixed in the half-concentrated Karnovsky solution[7] for 30 min under rotation at room temperature. The eye was divided perpendicular to the optical axis (just behind the ciliary body) with a razor-blade. The fundus, separated from the vitreous body, was cut into small (2 × 3 mm) pieces (tapetum lucidum and tapetum nigrum), and fixed for a further 90 min in the same fixative, but at 0–4 °C. After rinsing in 0.1 M sodium cacodylate + 7% sucrose, buffered at pH 7.4, the tissue was fixed for 60 min in 2% osmium tetroxide, contrasted in 0.1% uranyl acetate for 30 min, and dehydrated with ethanol/propylene oxide and embedded in Epon.

Ultrathin sections through the retina and adjcent tissue were mounted on silver grids, and coated with Ilford L4 emulsion by the loop method[8]. After an exposure time of between 4 and 28 days at 4 °C, the emulsion was developed with Kodak D 19 for 5 min at 20 °C, and fixed for 5 min with Kodak rapid fixer. The sections were stained with lead citrate and examined with a Philips EM 300 electron microscope.