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GLUCURONIDATION OF 1-NAPHTHOL IN THE RAT INTESTINAL LOOP

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Abstract—The glucuronidation of 1-naphthol after its instillation into the intestinal lumen was studied in closed or single pass perfused jejunal loops by measuring the appearance of 1-naphthol glucuronide in the intestinal lumen and in venous blood. Within 30 min 69% of 1-naphthol instilled into the jejunal loop was metabolized almost exclusively to the glucuronide; 49% was in venous blood and 20% in the intestinal lumen. After instillation of 1-naphthol glucuronide, 59% was left in the intestinal lumen and 20% appeared in venous blood. When the intestine was perfused with 1-naphthol, 91% of the naphthol absorbed from the intestinal lumen was glucuronidated at a rate of 6·7 nmole/min/g tissue; 82% of the glucuronide was released into the blood under steady state conditions. The results demonstrate the suitability of this system for studies on drug metabolism in intact intestinal tissue.

Many investigators have demonstrated UDP-glucuronyltransferase activity* in the intestinal mucosa using a variety of drugs and hormones [4–11]. We recently reported a simple and accurate method of determining glucuronidation rates in isolated perfused rat liver using 1-naphthol as substrate [12–14]. The intestine may contribute considerably to the overall glucuronidation of drugs or hormones in the organism. Moreover this metabolic pathway may play an important role in the absorption of drugs and other xenobiotics. The method recently used for the liver has therefore been extended to intact intestinal tissue in order to estimate glucuronidation rates under controlled experimental conditions.

Previous absorption studies have demonstrated the high functional integrity of the closed or perfused intestinal loop system [15, 16]. As in the case of the hepatocyte where glucuronides are released both into the bile and the blood, the epithelial cell of the intestinal mucosa also releases glucuronides into two different compartments namely the intestinal lumen and the blood. Since the venous blood from the perfused loop was completely collected it was possible to obtain a balance sheet of the products. The absorption of 1-naphthol glucuronide from the intestinal lumen into the blood was also studied because of the importance of this process during enterohepatic circulation.

MATERIALS AND METHODS

Chemicals were obtained from the following sources: 1-(1-^{14}C)-naphthol (20·8 mCi/m-mole) from The Radiochemical Center (Amersham); 1-naphthol glucuronide and 1-naphthol sulfate from Sigma Chemical Co. (St. Louis); β-glucuronidase (sulfatase-free) from Serva (Heidelberg).

^{14}C-1-Naphthol glucuronide was isolated from the perfusion medium of rat livers perfused with ^{14}C-1-naphthol [12–14]. After removal of the erythrocytes from the medium by centrifugation the radioactive metabolites were separated by thin-layer chromatography on cellulose plates (Merck, Darmstadt) developed in ethanol–1 M ammonium acetate (9:1, v/v). The peak corresponding to 1-naphthol glucuronide was extracted and its identity verified by its fluorescence spectrum and by hydrolysis to 1-naphthol with β-glucuronidase.

Preparation of the rat intestinal loop. Male Wistar rats (300–350 g) fed *ad lib.* on a standard diet containing 20% protein (Altromin, Lage-Lippe, Germany) were used. The intestinal loop was prepared under urethane anesthesia as previously described in detail [15, 16]. A jejunal loop about 22 cm from the *flexura duodenojejunalis* and about 5 cm long was selected and separated from the neighbouring loops. The vein collecting the blood from the jejunal loop was cannulated and blood was collected after passage through an optical drop recorder. A constant blood flow of about 1 ml/min per g tissue was usually reached. The lost blood was constantly replenished by infusion of heparinized blood through the jugular vein.

In the closed loop experiments 0·2 mM ^{14}C-1-naphthol or ^{14}C-1-naphthol glucuronide (0·5 ml in isotonic saline buffered with 0·07 M Soerensen phosphate buffer pH 6·8) was instilled into the lumen of the closed loop by injection. Naphthol was dissolved in 0·5% (v/v) ethanol (final concentration). At the end of the experiments (after 30 min) the luminal contents were collected, the intestinal loop washed with buffered saline and weighed. The mucosa was scraped off with a spatula and its wet weight also determined. The mucosa was homogenized in 4 vol 0·25 M sucrose and centrifuged at 100,000 *g* for 30 min. The supernatant was collected and the sediment dissolved in Protosol (NEN, Boston, Mass.) for the determination of radioactivity. The wet weight of an intestinal loop of about 5·4 cm was 400 ± 18 mg and of its corresponding mucosa 290 mg.

In the single pass perfusion experiments the loop was cannulated at both ends and washed with iso-

* There is accumulating evidence for a multiplicity of UDP-glucuronyltransferases [1–3]. Since the number of substrate specific forms of this enzyme is uncertain, in this paper UDP-glucuronyltransferase refers only to the enzyme catalyzing the glucuronidation of 1-naphthol.

tonic saline. The loop was then perfused at a rate of 0·1 ml/min with 65 μM naphthol in buffered saline containing 0·5% ethanol. The outflowing perfusate and the intestinal venous blood was collected for the determination of 1-naphthol and 1-naphthol glucuronide. Free naphthol and its glucuronide was also determined in mucosal fractions as described above for the closed loop experiments.

Determination of 1-naphthol glucuronide and free 1-naphthol. For the determination of naphthol and its conjugates a rapid radio-assay was used [14]. In this method the unmetabolized ^{14}C-1-naphthol was extracted into a toluene scintillation fluid (containing 0·5% (w/v) PPO and 0·05% (w/v) POPOP), whereas the conjugates remained in the aqueous phase with their radioactivities quenched. The sum of free naphthol and all of its metabolites was determined in a dioxane based scintillation fluid [15]. The identity of naphthol and its metabolites in venous blood plasma and the luminal contents was verified by thin-layer chromatography on cellulose plates (Merck, Darmstadt) developed in ethanol–1 M ammonium acetate (9:1, v/v). Since 1-naphthol glucuronide was the only major metabolite, it could be calculated from the difference of the radioactivities determined in the toluene and dioxane scintillation fluids. Quenching was monitored by the addition of an internal standard. Since unmetabolized naphthol was almost entirely extracted into the toluene scintillation fluid (partition between the aqueous phase and the toluene phase = 1:60) the amount of naphthol remaining in the aqueous phase could be neglected. However a 3% extraction of 1-naphthol glucuronide into the toluene phase was taken into account since usually low amounts of naphthol had to be determined in the presence of high amounts of glucuronides. In control experiments it was found that naphthol glucuronide was excluded from erythrocytes. However free naphthol was mainly bound to erythrocytes presumably to the plasma membrane. The partition of 1-naphthol between blood cells and plasma was found to be about 4:1 within a range of 1–5 nmoles/ml plasma usually obtained in the experiments. Within this range free naphthol could be calculated from its concentration in plasma. 1-Naphthol could not be determined accurately below a concentration of 1 nmole/ml plasma.

The contact of 1-naphthol with soft plastic material should be avoided because of its considerable uptake.

RESULTS

Instillation of 1-naphthol into the closed jejunal loop. When 100 nmoles 1-naphthol was instilled into the jejunal loop, naphthol glucuronide could be detected in venous blood after 1 min (Fig. 1). The rate of naphthol glucuronide formation was maximal after 4 min reaching a concentration of 31 nmoles/ml plasma. The rate decreased afterwards with 1st order kinetics ($t/2$ = 7 min). Free naphthol also appeared in blood up to 2 min with the same rate as naphthol glucuronide (maximal concentration of 1-naphthol = 5 nmoles/ml plasma). The rate thereafter declined with $t/2$ = 3 min. Free naphthol was hardly detectable in intestinal venous blood after 10 min. After 30 min about 7% of the free naphthol was still present in the luminal contents together with 20% of the naphthol glucuronide. During the same time period 49% of the naphthol glucuronide appeared in venous blood. Naphthol was metabolized almost exclusively to the glucuronide. Only an insignificant amount of naphthol sulfate was found after thin-layer chromatography of the blood plasma with the system described in Methods. This system clearly separates 1-naphthol glucuronide, 1-naphthol sulfate and 1-naphthol with R_f-values of 0·2, 0·8 and 0·9, respectively.

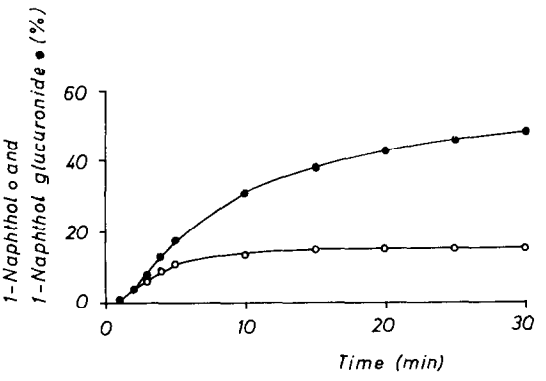

Fig. 1. Appearance of 1-naphthol glucuronide (●) and free 1-naphthol (○) in intestinal venous blood after instillation of 100 nmoles (0·6 μCi) ^{14}C-1-naphthol into the closed jejunal loop. The mean of six experiments is given.

Virtually 100% of the glucuronide was hydrolyzed with β-glucuronidase in the lumen but only 60–75% could be split in intestinal venous blood under the same conditions suggesting an inhibition of β-glucuronidase. The inhibition was about 25% after the addition of plasma obtained before instillation of 1-naphthol into the closed loop. Addition of plasma obtained during active glucuronidation (20–30 min after instillation of 1-naphthol) caused an about 40% inhibition of β-glucuronidase (Table 1).

Instillation of 1-naphthol glucuronide into the closed intestinal loop. When naphthol glucuronide was instilled into the closed intestinal loop the conjugate was absorbed from the intestinal lumen into venous blood (Fig. 2). An initial rapid rate was followed by an almost constant rate. After 30 min 59% of the glucuronide was still present in the intestinal

Table 1. Hydrolysis of 1-naphthol glucuronide by β-glucuronidase in the presence of blood plasma

Experimental conditions	Addition of rat plasma	^{14}C-1-Naphthol glucuronide (cpm)	hydrolyzed (%)
A	–	1470	100
	+	1100	75
B	–	4500	100
	+	2570	57

Venous blood from an intestinal loop was obtained before (A) and during (B) glucuronidation of 1-naphthol by the intestinal mucosa. 0·05 ml of the corresponding plasma was incubated in 1·0 ml with ^{14}C-naphthol glucuronide (0·2 mM) and 2 mg β-glucuronidase in 0·1 M acetate buffer pH 4·5 for 18 hr at 37°. Free naphthol was determined as described in Methods. 95–100% of the added glucuronide was hydrolyzed when no plasma was present. Results are the average of three experiments.

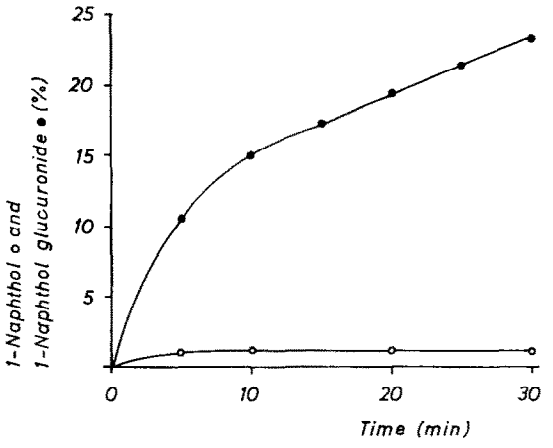

Fig. 2. Appearance of 1-naphthol glucuronide (●) and free 1-naphthol (○) in intestinal venous blood after instillation of 100 nmoles (0·03 μCi) ^{14}C-1-naphthol glucuronide into the closed jejunal loop. The mean of three experiments is given.

lumen together with 3% free naphthol. Hydrolysis of naphthol glucuronide presumably by the gut flora was slow under our experimental conditions. This is supported by an experiment in which pooled luminal contents were incubated *in vitro* (Table 2). Only about 2% naphthol glucuronide was hydrolyzed by the luminal content within 0·5 hr assuming a linear hydrolysis rate. However, during the same period 23% of naphthol glucuronide was found in venous blood suggesting a transport of glucuronide from the intestinal lumen to the blood without hydrolysis by the gut flora and subsequent absorption of the aglycon by the epithelial cells.

Single pass perfusion of the intestine with 1-naphthol. When the intestinal loop (0·4 g) was perfused with 65 μM 1-naphthol at a rate of 0·1 ml/min the concentration of naphthol in the outflowing perfusate was found to be 37 μM. Constant rates of appearance of naphthol glucuronide were found in venous blood and in the perfusate from the intestinal loop (Fig. 3). Under these steady state conditions rates of glucuronidation and transport to the different compartments were calculated (Fig. 4). The sum of the appearance rates of naphthol and naphthol glucuronide (7·4 nmoles/min per g) closely resembled the disappearance rate of naphthol in the perfused loop (7 nmoles/min per g). 91% of naphthol was glucuronidated at a rate of 6·7 nmoles/min per g jejunum. This corresponds with 9·2 nmoles/min per g mucosa wet weight since 0·29 g mucosa was usually obtained from 0·4 g jejunum (as given in Methods).

Table 2. Hydrolysis of 1-naphthol glucuronide by the luminal content of intestinal loops

Time (hr)	1-Naphthol glucuronide (cpm)	1-Naphthol (cpm)
0	12,500	3900
5	10,450	5900
12	4600	10,750

Pooled luminal contents from experiments described in Figs. 1 and 2 were incubated at 37°. At different times samples were analyzed for naphthol glucuronide and naphthol as described in Methods.

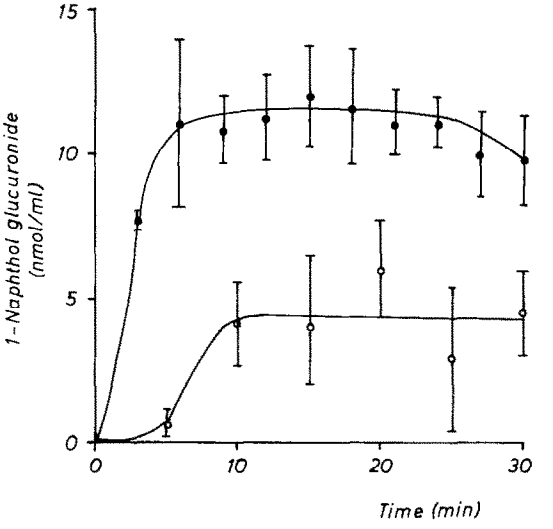

Fig. 3. Concentrations of 1-naphthol glucuronide in intestinal venous blood (●) and in the outflow of the perfused loop (○) after perfusion of the intestine with 65 μM 1-naphthol at a rate of 0·1 ml/min. The mean ± S.D. of six experiments is given.

The concentration of naphthol glucuronide in the cytoplasm of epithelial cells (the 100,000 *g* supernatant of the homogenate obtained from the intestinal mucosa) was similar to that determined in blood plasma after 30 min indicating that there was no accumulation of glucuronides within the epithelial cells. Free naphthol could also be detected in the mucosa mainly bound to the 100,000 *g* sediment.

DISCUSSION

^{14}C-1-Naphthol and its conjugates can be determined very simply by counting samples both in a dioxane and toluene based scintillation fluid [17]. This method which may be useful for the determination of other lipophilic drugs and their more hydrophilic metabolites [19] is only suitable when the partition of the substrate and its products between the aqueous and the toluene phase is known and almost exclusive; e.g. 1-naphthol is mainly extracted

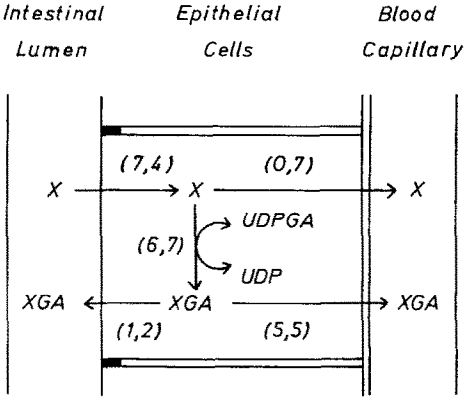

Fig. 4. Metabolism of 1-naphthol (×) by epithelial cells of rat intestine under steady state conditions (GA = glucuronic acid). Values in parentheses represent rates of naphthol or naphthol glucuronide metabolized or transported to the given compartment in nmoles/min per g intestine. Rates were calculated under the steady state conditions listed in Fig. 3 as described in the text.

into the toluene phase, only 2% remaining in the aqueous phase, whereas only 3% of 1-naphthol glucuronide can be detected in the toluene phase under our conditions. The method previously used to determine glucuronidation rates in the isolated perfused liver [12–14] has now been applied to the perfused intestinal loop. With the intestine the analysis was especially simplified since 1-naphthol is almost exclusively glucuronidated.

When the intestine was perfused with 65 μM 1-naphthol 1-naphthol glucuronide was released into the blood and the intestinal lumen under steady state conditions. Since no accumulation of glucuronide in the intestinal mucosa was found the rate of release corresponded with the rate of glucuronide formation. It was found to be about 7 nmoles/min per g intestine (Fig. 4) or 10 nmoles/min per g mucosa.

In liver a quantitative comparison between glucuronidation rates in intact tissue and in microsomes has been attempted [13, 14]. Since the membrane-bound UDP-glucuronyltransferase (1-naphthol as substrate) can be activated about 10-fold by various treatments altering the membrane structure [2, 20–23] it was interesting to know in which form the enzyme was acting within the cell. It was possible to demonstrate that the maximally activated form is not operating *in vivo* i.e. the enzyme is mostly latent or constrained *in vivo*. In the intestine such a comparison would be particularly valuable since UDP-glucuronyltransferase in native microsomes obtained from the intestinal mucosa could not be further activated by treatments known to stimulate the enzyme in liver e.g. detergents, sonication or trypsin (unpublished results). With a comparison of naphthol glucuronidation in intact mucosal tissue and in the corresponding microsomes it may be possible to decide whether the mucosal enzyme is activated during the isolation procedure by intestinal proteases or is less constrained in the intestine than in liver.

In liver tissue the level of UDP-glucuronic acid did not decrease even during prolonged glucuronidation at maximal rates [14] indicating that the regeneration of this nucleotide could not be exhausted. In the intestinal mucosa the formation of UDP-glucuronic acid must also be high since a constant and high rate of glucuronide formation was maintained by this tissue. The consistent finding of an inhibition of β-glucuronidase in venous blood (especially during active glucuronidation, Table 2) may be caused by the release of metabolic products of UDP-glucuronic acid e.g. D-glucaric acid which is known to be a potent inhibitor of β-glucuronidase.

It is conceivable that the highly polar glucuronides are transported by carrier proteins through the plasma membrane of hepatocytes into the bile and the blood. It has been shown that in the rat glucuronides above a threshold molecular weight of 325 are excreted predominately into the bile [24]. The epithelial cell of the intestinal mucosa also excrete glucuronides into two different compartments: the blood and the intestinal lumen. However, the regulation of this excretion is still obscure. Similar to the liver where glucuronides can be absorbed from the blood and excreted into the bile, 1-naphthol glucuronide was mainly absorbed unchanged from the intestinal lumen and excreted into the blood. The absorption of the unchanged glucuronide may contribute to a considerable extent to the enterohepatic circulation of drugs in addition to the hydrolysis of glucuronides by the gut flora and subsequent absorption of the aglycone.

The significance of intestinal glucuronidation in the detoxification of xenobiotics and of hormones is poorly understood. It may have considerable pharmacological implications in the absorption of drugs. Several phenolic drugs, e.g. morphine, are said to be poorly absorbed orally while their *o*-methylated congeners are not. Glucuronidation may provide an alternate explanation for the low levels of free drug obtained. It is also interesting to note that highly lipid-soluble compounds such as 1-naphthol may be transported in blood bound to the erythrocyte plasma membrane.

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REFERENCES

1. G. J. Dutton, in *Glucuronic Acid, Free and Combined* (Ed. G. J. Dutton) p. 185. Academic Press, New York (1966).
2. K. W. Bock, W. Fröhling, H. Remmer and B. Rexer, *Biochim. biophys. Acta* **327**, 46 (1973).
3. D. Zakim, J. Goldenberg and D. A. Vessey, *Biochim. biophys. Acta* **309**, 67 (1973).
4. F. Zini, *Sperimentale* **102**, 40 (1952).
5. K. J. W. Hartiala, *Acta physiol. scand.* Suppl. **114**, 20 (1954).
6. D. Schachter, D. J. Kass and T. J. Lannon, *J. biol. Chem.* **234**, 201 (1959).
7. R. Herz, Jr., D. F. Tapley and J. E. Ross, *Biochim. biophys. Acta* **53**, 273 (1961).
8. I. H. Stevensen and G. J. Dutton, *Biochem. Pharmac.* **82**, 330 (1962).
9. T. A. Miettinen and E. Leskinen, *Biochem. Pharmac.* **12**, 565 (1963).
10. O. Hänninen, A. Aitio and K. Hartiala, *Scand. J. Gastroenterol.* **3**, 461 (1968).
11. W. H. Barr and S. Riegelman, *J. Pharmaceut. Sci.* **59**, 154 and 164 (1970).
12. K. W. Bock and W. Fröhling, *Naunyn-Schmiedebergs Arch. Pharmac.* **277**, 103 (1973).
13. K. W. Bock, *Naunyn-Schmiedebergs Arch. Pharmac.* **283**, 319 (1974).
14. K. W. Bock and I. N. H. White, *Eur. J. Biochem.* **46**, 451 (1974).
15. D. Winne, *Naunyn-Schmiedebergs Arch. Pharmac.* **254**, 199 (1966).
16. H. Ochsenfahrt and D. Winne, *Naunyn-Schmiedebergs Arch. Pharmac.* **264**, 55 (1969).
17. G. W. Lucier, O. S. McDaniel and H. B. Matthews, *Archs Biochem. Biophys.* **145**, 520 (1971).
18. G. A. Bray, *Analyt. Biochem.* **1**, 279 (1960).
19. R. Hess, D. Leschem, Hj. Teschemacher and A. Herz, *Eur. J. clin. Pharmac.* **5**, 104 (1972).
20. K. K. Lueders and E. L. Kuff, *Archs Biochem. Biophys.* **120**, 198 (1967).
21. E. Halac and A. Reff, *Biochim. biophys. Acta* **139**, 328 (1967).
22. G. J. Mulder, *Biochem. J.* **117**, 319 (1970).
23. O. Hänninen and R. Puukka, *Suom. Kemistil.* **43**, 451 (1970).
24. M. M. Abou-El-Makarem, P. Milburn, R. L. Smith and R. T. Williams, *Biochem. J.* **105**, 1269 (1967).