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CONJUGATION OF 1-NAPHTHOL AND TRANSPORT OF 1-NAPHTHOL-CONJUGATES IN THE VASCULARLY PERFUSED SMALL INTESTINE OF THE MOUSE

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Abstract—A method is described which allows the simultaneous vascular and luminal perfusion of the murine small intestine. This preparation was used for the investigation of 1-naphthol conjugation in the gut and the sidedness of conjugate release. The viability of this preparation can be maintained for more than 1 hr as indicated by morphological controls, measurement of tissue metabolism and the transport of 3-*O*-methyl-glucose against a concentration gradient. When 100 μM 1-naphthol was administered on the luminal side, it was conjugated at a constant rate, yielding 1-naphthyl-glucuronide and 1-naphthyl-sulfate in a molar ratio of 1:2. Both metabolites were excreted into the blood at the contraluminal side of the epithelium. The results are discussed with respect to the sidedness of intestinal transport systems for anionic conjugates of xenobiotics and drugs.

Metabolism of orally ingested drugs or xenobiotics may occur to a large extent in the epithelium of the gastrointestinal tract. This was shown in dogs for isoproterenol [1–4], a drug which is mainly converted into its glucuronide by hepatic enzymes when it is given intravenously, whereas sulfation in the gastrointestinal tract prevails after oral administration. The phenolic compound 1-naphthol, which is formed upon hydrolysis of the pesticide naphthyl-*N*-methyl-carbamate in the gut, is converted into its glucuronide exclusively in everted sacs as well as in vascularly perfused preparations of rat small intestine [5–9]. In man and some other species—in contradistinction to the rat—intestinal sulfation of phenols is a relevant additional detoxication pathway (for a review see [10]). In preliminary experiments we found that the gastrointestinal tract of the mouse has a high capacity for 1-naphthol sulfation. Therefore this species was chosen for our experiments.

Drug conjugates which are formed in the liver can be released over the biliary or venous pole of the hepatocyte depending on their physicochemical properties, as has been extensively studied. The enterocyte shows an even more pronounced morphological polarity than the hepatocyte, but only limited information is available on the fate of polar drug conjugates which are formed by the mucosa. Whether these organic anions leave the epithelium either to the mucosal or to the blood side using an anion transport system, or whether they leave the epithelium in both directions without any preference are unanswered questions.

One experimental approach for the investigation of conjugate release was the use of *in vitro* models with intact polarity and transport functions of the epithelium. A means of achieving this was by using vascularly and luminally perfused preparations which had been established for rat small intestine. However, as sulfoconjugation activity is very low in this species, it was necessary to develop a method for an open vascular and luminal perfusion of the mouse small intestine.

The purpose of this contribution is to describe this method, to demonstrate its suitability for studying the intestinal sulfoconjugation of foreign compounds and to answer the question whether the hydrophilic anionic metabolites, which are formed inside the epithelium, are excreted preferentially to the luminal or the contraluminal side.

MATERIALS AND METHODS

Chemicals. $H_2{}^{35}SO_4$, carrier-free, 37 MBq/ml, was purchased from New England Nuclear (Dreieich, F.R.G.) and was diluted 1:10 with distilled water to give a stock solution. Radioactive 1-[^{14}C]naphthol (specific activity 0.74 GBq/mmole) and 3-*O*-methyl-D-[U-^{14}C]-glucose (specific activity > 1.85 GBq/mmole) were purchased from the Radiochemical Centre, Amersham (Braunschweig, F.R.G.).

Since 1-naphthol decomposes in the presence of oxygen, stock solutions (0.1 M) were made by dilution to a specific activity of 12.3 MBq/mmole with cold 1-naphthol in absolute ethanol and were kept under an atmosphere of nitrogen at −18°. Radioactive 2-[^{3}H]-naphthol-orange was a generous gift from Dr. F. Lauterbach (Ruhr-Universität, Bochum, F.R.G.).

Ficoll 70 was from Pharmacia Fine Chemicals (Freiburg, F.R.G.); phlorizin from Sigma (München, F.R.G.); and penicillin G, streptomycin, heparin-Na (> 150000 IE/g), cyproheptadine, pluronic F68, 1-naphthol and 3-*O*-methyl-glucose (3-*O*-MG) were from Serva (Heidelberg, F.R.G.). All other chemicals were obtained from Merck (Darmstadt, F.R.G.). All substances were of the highest analytical grade available.

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Enzymes. β-Glucuronidase (10–20 U/g) EC 3.2.1.31 from bovine liver (sulfatase free) and β-glucuronidase (5 U/ml)/arylsulfatase (2.5 U/ml) EC 3.2.1.31/EC 3.1.6.1. (= glusulase) from *Helix pomatia* were obtained from Serva. The activities and purity are given as stated by the supplier. β-Glucuronidase was dissolved in acetate buffer to give a solution of 1 mg/ml.

Animals. Male NMRI-SPF mice (25–30 g) were purchased from Wiga (Sulzfeld, F.R.G.). The animals had free access to tap water and to a commercial pelleted diet (Altromin®). Food, but not water, was removed 12 hr before the experiment.

Perfusion media. The vascular perfusion medium consisted of a modified Krebs–Ringer buffer and washed human erythrocytes. One hundred ml of the final medium contained 30 ml of packed erythrocytes, 2.8 g Ficoll 70, 60 mg penicillin G, 7.5 mg streptomycine sulfate, 1 mg heparin-Na, 4 μg cyproheptadine, 60 mg Pluronic F68, which is a non-ionic surfactant [11], 10 nmole dexamethasone 21-acetate and 1 mmole glucose. Human erythrocytes were prepared from fresh unit bags. The composition of the modified Krebs–Ringer buffer was as follows: 120 mM NaCl, 4.5 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM Na_2HPO_4, 1.0 mM $MgSO_4$ and 1.25 mM $CaCl_2$. The pH was kept at 7.5 by gassing with a mixture of moist 95% O_2–5% CO_2 in a rotating flask oxygenator.

The luminal perfusion medium was Dulbecco's phosphate buffered saline (PBS), pH 7.5.

Perfusion apparatus. The whole assembly was placed in a chamber fitted with a thermostat and kept at 37°. Vascular perfusion was performed at a constant pressure of 80 mm Hg, yielding a flow rate between 2.5 and 3 ml/min. Single pass perfusion was used for both the lumen and vascular bed. The animal was placed on a slanting table so that the intestine hung down into an organ bath filled with PBS.* All glassware which was in contact with the vascular perfusion medium was silicone-coated in order to minimize haemolysis. Samples were taken from the venous and luminal outflow cannulae at fixed time intervals. Flow rate and sample volume were determined by weighing.

Surgical procedure. Animals were anaesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg) and the intestine was prepared according to Dubois *et al* [12]. Modifications were made in order to accommodate for the smaller size of the mouse compared to the rat. The entire small intestine was perfused from the ligamentum of Treitz to the ileocecal valve.

Electron microscopy. Samples (30) for electron microscopy were taken after a perfusion period of 60 min. Specimens were fixed with glutaraldehyde and freeze-fractures were prepared using conventional techniques. Specimens of various gut segments were inspected in order to confirm good preservation along the entire length of the tissue.

Analytical methods. Glucose and l-lactate were determined with commercial test kits (Merckotest-glucose and Boehringer-lactate). Results were corrected for erythrocyte metabolism.

Naphthol conjugates were determined by several methods.

Total conjugates and free naphthol were quantitated according to Bock and White [13] by determining total radioactivity in Bray scintillator and toluene-extractable radioactivity in toluene scintillator, the difference being total conjugates.

The metabolites were identified by thin-layer chromatography (TLC) and by enzymatic degradation. For TLC, 1-naphthol, 1-naphthyl-glucuronide and 1-naphthyl-sulfate were spotted on silica gel 60 plates (Merck 11798) and the chromatograms developed with *n*-propanol/1 M ammonium acetate (6:1 v/v) at about 20°. R_f values of 0.90, 0.68 and 0.31 were found for the three compounds (order as above).

For enzymatic degradation, samples of the venous effluent were centrifuged and 450 μl of each supernatant were buffered with 50 μl of 1 M acetate buffer, pH 4.5 (acetic acid). Ten μl of Krebs–Ringer buffer (control), or glucuronidase- or glusulase-suspension were added. After an incubation period of 18 hr at 20°, a 100 μl aliquot of incubate was added to 10 ml of Bray scintillator for the determination of total radioactivity, and another 200 μl of incubate was added to 10 ml of toluene scintillator for the determination of toluene-soluble radioactivity. Non-enzymatic hydrolysis did not occur in the control incubates.

Experiments with $^{35}SO_4$. In experiments with $^{35}SO_4$, the free [^{35}S]sulfate was precipitated by the addition of 100 μl of 0.25 M Na_2SO_4 and a subsequent addition of 100 μl 0.5 M $BaCl_2$ to 400 μl of medium. The precipitate was spun down and 400 μl of the supernatant was counted. Control experiments revealed that no conjugate co-precipitated whereas free sulfate was quenched below the detection limit of the counter.

Radioactivity measurements. Radioactivity was measured in a Packard Tri-Carb 3380 liquid scintillation counter using standard Bray and toluene scintillators. Quench corrections were made by external standardization.

RESULTS

The viability of the preparation was controlled by means of three criteria, namely the preservation of an intact morphology, metabolism of glucose, and the ability to actively transport 3-*O*-MG.

After 1 hr of perfusion the morphology of the epithelium did not show any abnormality compared to non-perfused controls. Mitochondria were neither swollen nor did vacuolization occur. Golgi apparatus had normal vesiculations.

Glucose uptake from blood was 6.1 ± 1.5 μmole/min per g dry weight (n = 11). The fraction of glycolysis, a relevant parameter of tissue viability, was 44.7 ± 5.4% (n = 11). This was in good agreement with the *in vivo* and *in vitro* values found by others in rat small intestine [14–16]. Glucose consumption and lactate production remained constant for 1 hr. Glucose did not leak into the luminal perfusate even

* Abbreviations: PBS = Dulbecco's phosphate-buffered saline; 3-*O*-MG = 3-*O*-methyl-glucose, 2-naphthol-orange = sodium azo-β-naphthol-sulfanilate, PAPS = 3'-phosphoadenosine-5'-phosphosulfate.

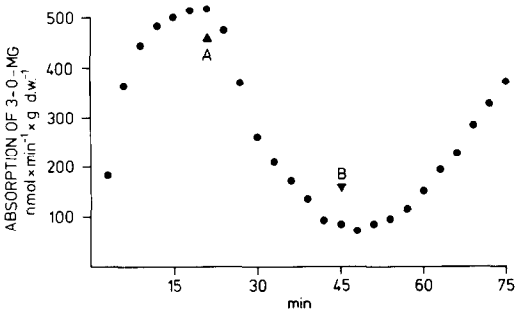

Fig. 1. Absorption of 3-*O*-MG by the perfused mouse small intestine. Phlorizin (0.1 mM) was present in the luminal medium during the time between the arrows.

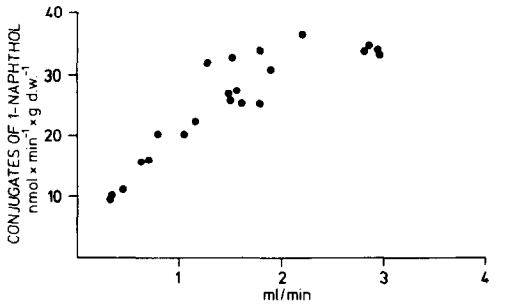

Fig. 3. Dependence of the release of 1-naphthol polar metabolites into the vascular perfusate on the vascular flow rate.

when the venous glucose concentration was raised to 13 mM. Lactate concentrations in the luminal perfusate were close to the detection limit and never exceeded 4% of the total lactate released (5.2 ± 2.3 μmole/min per g dry weight; $n = 11$).

'Active transport', i.e. transport against a concentration gradient, of sugars and amino acids is indicative of an intact functioning of *in vitro* preparations. Therefore the transport of 3-*O*-MG, an actively transported non-metabolized glucose analogue [17], was measured. Two types of experiment were carried out. In the first the luminal and vascular medium contained 1 mM 3-*O*-MG. Tracer [^{14}C]-3-*O*-MG was added to the luminal medium and its transport into the blood was measured. The rate of appearance in the blood of 3-*O*-MG was 622 ± 112 nmole/min per g dry weight. It could be inhibited by phorizin (Fig. 1). In order to demonstrate that 3-*O*-MG was transported uphill, further experiments were conducted in which 2.3 mM radiolabelled 3-*O*-MG of the same specific activity was present in both the vascular and luminal perfusion media. When the luminal perfusion was stopped for 10 min, there was a decrease in the luminal 3-*O*-MG concentration to 0.9 ± 0.2 mM ($n = 3$). Thus a concentration gradient from blood to lumen (3:1) was established.

Conjugation of 1-naphthol and release of its conjugates

When the luminal perfusion fluid was replaced by a solution containing radioactive 1-naphthol, an increasing amount of polar metabolites appeared in the venous effluent, reaching a steady state after 7–10 min (Fig. 2). In contrast, polar metabolites were not detected in the luminal effluent.

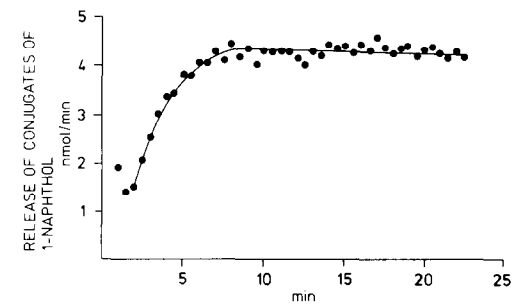

Fig. 2. Formation of polar metabolites of 1-naphthol in the vascularly perfused small intestine at a saturating substrate concentration of 0.1 mM.

The rate of release of 1-naphthol polar metabolites into the vascular perfusate depended on the vascular flow rate. When the flow rate was increased by changing the perfusion pressure, a concomitant increase in output of metabolites was observed. The excretion rate reached a steady state at a perfusion rate of 2.3–3 ml/min (Fig. 3). This perfusion rate corresponded to an arterial perfusion pressure of 60–80 mm Hg. The concentration of the metabolites in the venous effluent decreased simultaneously. The maximal production rate of polar metabolites was 33 ± 7 nmole/min per g dry weight small intestine ($n = 15$). This was equivalent to a detoxication capacity of roughly 1 mg of 1-naphthol conjugated per animal (30 g) per day.

Two independent methods were used to identify the polar products which were formed from 1-naphthol by the epithelium. When the supernatant of the venous effluent was separated on TLC plates, radioactivity co-migrated with 1-naphthyl-sulfate, 1-naphthyl-glucuronide and free 1-naphthol. The distribution between the two conjugates was 33 ± 3% for the glucuronide and 66 ± 2% for the sulfate ($n = 3$). In order to confirm this result, samples of the venous supernatant were subjected to enzymatic degradation. Glusulase hydrolysed 98 ± 1% of the conjugates, whereas glucuronidase cleaved only 33 ± 4%. This was in good agreement with the TLC results.

It has been reported that sulfate supply is the limiting factor of the sulfoconjugation capacity *in vivo* [18]. Concerning intestinal sulfoconjugation,

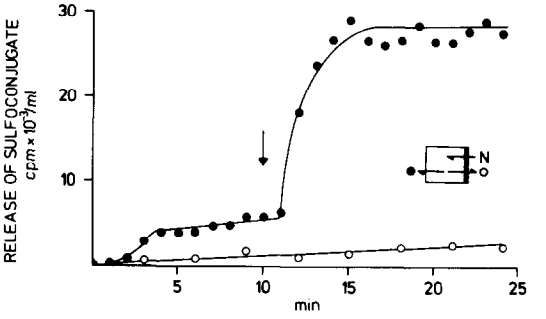

Fig. 4. Conjugate release into the vascular (●) and luminal (○) medium before and after the addition of 1-naphthol (0.1 mM) to the luminal medium at the time shown by the arrow.

no information is available about the sources of sulfate. Therefore we investigated the importance of luminal or vascular inorganic sulfate for the conjugation of 1-naphthol. In these experiments sulfate was omitted from the media during the first 30 min of perfusion and was given in a 1 mM concentration in the luminal or vascular perfusion fluid thereafter. This protocol revealed a high inter-individual variation in the dependence of conjugation activity on exogenous sulfate. In some animals, vascularly as well as luminally administered sulfate increased the rate of naphthyl-sulfate synthesis considerably, whereas almost no effect was seen in others. The median increase amounted to 22% ($n = 10$), with a range from 5 to 80%.

The incorporation of exogenous sulfate into the intracellular PAPS pool offered the opportunity for labelling of sulfoconjugates by adding radioactive sulfate to the perfusion media. Since the radioactivity was diluted by inactive endogenous sulfate and since the contribution of exogenous sulfate to the total conjugation capacity was variable and might be low, only a tracer amount of radioactive sulfate was used. In these experiments, $MgSO_4$ was replaced by $MgCl_2$ in the perfusion media. Under this experimental condition, non-precipitable radioactivity was released to the luminal and vascular media in the absence of 1-naphthol (Fig. 4). This indicated the formation of an unknown endogenous sulfo-conjugate. The addition of unlabelled 1-naphthol to the luminal medium caused a five-fold increase of non-precipitable radioactivity in the vascular, but not in the luminal, effluent. The ^{35}S-activity in the vascular effluent co-migrated with 1-naphthyl-sulfate on TLC plates.

Transport of 2-naphthol-orange

From the results reported above, it follows that the brush-border membrane of the mouse small intestine is impermeable to 1-naphthyl-sulfate. In the guniea-pig ileum, however, sulfonic acids like sulfanilic acid, phenol red and 2-naphthol orange have been shown to be secreted transcellulary from blood to lumen [19]. These organic anions are structurally related to 1-naphthyl-sulfate and one might speculate that they share the same transport mechanism. If this were the case, no transport of 2-naphthol-orange from blood to lumen would be seen in the mouse small intestine. However, when tritiated 2-naphthol-orange (1 nmole/ml, Fig. 5) was dissolved in the luminal or vascular perfusion medium,

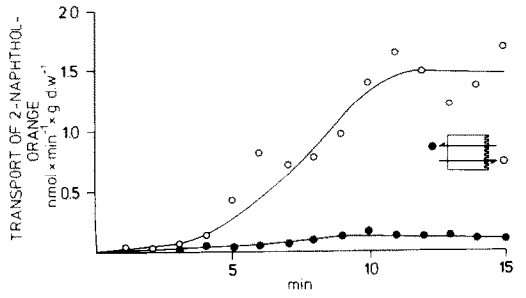

Fig. 5. Transport of 2-naphthol-orange from lumen to blood (●) and from blood to lumen (○).

a steady-state movement of 1.4 ± 0.5 nmole/min per g dry weight ($n = 5$) from blood to lumen was measured, whereas movement from lumen to blood was lower by an order of magnitude (0.09 ± 0.02 nmole/min per g dry weight; $n = 5$).

DISCUSSION

The vascularly perfused mouse small intestine described in this paper remains viable for at least 1 hr. Morphological examination did not reveal any signs of deterioration after this time. Furthermore, the state of the glucose metabolism is close to that under *in vivo* conditions. The fraction of glycolysis amounted to 45%, which is in good agreement with data from the rat small intestine measured *in vitro* [14, 15], as well as *in vivo* [16]. Furthermore, the vascularly perfused mouse small intestine transports 3-*O*-MG, a non-metabolizable glucose analogue, against a concentration gradient, which is the strongest argument in favour of the functional integrity of the preparation.

The preparation was used for the investigation of 1-naphthol metabolism in, and release of its metabolites from, the epithelium. The polar products which were formed were identified as 1-naphthyl-sulfate and 1-naphthyl glucuronide. In mouse the sulfation exceeded glucuronidation by a factor of two, whereas in rat small intestine Pekas *et al.* [5–7], Bock and Winne [8], and Josting *et al.* [9] found 1-naphthyl-glucuronide to be the only conjugation product. The endogenous source of sulfate can be supplemented by luminal or blood inorganic sulfate. The contribution of exogenous sulfate to the total sulfation capacity varied considerably between individual animals. This result indicates the existence of endogenous anaplerotic reactions for PAPS which work at a different rate in different animals. The pool size of precursor sulfur, which is required to supply the conjugation for 1 hr, was estimated from the conjugation rate to be about 300 nmole in the whole small intestine. A 1 mM intracellular concentration of free sulfate or cysteine would exceed this amount, if a wet weight of active tissue in the range of 0.5–1 g were assumed. The identity of the endogenous precursor(s) of PAPS is as yet unrevealed.

The rate of metabolite output in the preparation was correlated to the vascular flow rate. This phenomenon has been described in rats for the absorption of several drugs [20], but to our knowledge it has not yet been shown for compounds which are formed intracellularly.

1-Naphthol, which is a highly lipid-soluble compound, enters the mucosa by diffusion through the lipid phase of the cell membrane. The conjugates, which are formed intracellularly, are negatively charged at physiological pH values. Their lipid solubility is therefore very low compared to the parent compound and their release from the cells can be expected to be carrier-mediated. Intestinal organic anion transport systems have been described which carry their substrates from the luminal to the contraluminal side of the epithelium [21], as well as vice versa [19]. One might speculate that the conjugates share one or other of these pathways when they

leave the cells. In everted sacs of rat small intestine, Pekas noted that a concentration gradient of polar metabolites between the serosal and the mucosal side is established (ratio 7:1) by the epithelium [7]. In our preparation, no metabolites were released into the lumen at all. This difference is possibly due to the fact that back-diffusion of metabolites via the paracellular pathway is higher in everted sacs than in vascularly perfused gut preparations. The findings of Pekas as well as ours both may be explained by differences in permeability of the basolateral and brush-border membranes for the conjugation products. In our preparation this holds for the sulfoconjugate as well as for the glucuronide, which is the only product in the rat.

The lack of sulfoconjugate release to the lumen led us to compare the transport of a similar organic sulfonate, 2-naphthol-orange, a substance which is actively secreted from blood to lumen in guinea-pig ileum, in the mouse small intestine. This transport requires uptake at the basolateral membrane and release over the brush-border membrane in sequence. Since, in contrast to 2-naphthol-orange, 1-naphthyl-sulfate was not released from the intracellular space to the lumen in mouse small intestine, one can conclude that 1-naphthyl-sulfate does not share the carrier for 2-naphthol-orange in the luminal membrane. This does not exclude the possibility that serosal uptake of 2-naphthol-orange and the serosal release of 1-naphthyl-sulfate are mediated by the same anion-transport system. Further experiments may reveal whether all phenolic sulfate esters are released to the blood side, or whether some of them enter the luminal compartment as well.

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