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3609

Enterohepatic recycling of phenolphthalein, morphine, lysergic acid diethylamide (LSD) and diphenylacetic acid in the rat
Hydrolysis of glucuronic acid conjugates in the gut lumen

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1. Biliary elimination in female Wistar albino rats 3 h after i.p. injection of [^{3}H]phenolphthalein, [^{3}H]morphine, ^{14}C-LSD and [^{14}C]diphenylacetic acid was 90%, 45%, 75% and 57% respectively, predominantly as glucuronides.

2. Infusion of 3 h bile from the previous experiments into the duodena of bile-duct-cannulated animals demonstrated enterohepatic circulation, amounting in 24 h to 85%, 41%, 28% and 66% of the infused doses of the conjugates of phenolphthalein, morphine, LSD and diphenylacetic acid respectively.

3. Pretreatment with antibiotics to suppress intestinal microflora decreased this enterohepatic recirculation to 22%, 8·6% and 21% in 24 h for phenolphthalein, morphine and diphenylacetic acid glucuronides respectively. Antibiotic pretreatment did not influence the absorption and re-excretion of infused doses of the free aglycones, thus demonstrating the importance of bacterial β-glucuronidase hydrolysis of the biliary conjugates.

4. The extent of intestinal absorption of the aglycones after bacterial β-glucuronidase hydrolysis of the conjugates is related to their lipid-solubility as estimated by octan-1-ol: 0·1 M phosphate buffer partition ratios (P-values).

5. The persistence of compounds in the enterohepatic circulation is determined by the faecal and urinary elimination of the circulating compounds. Faecal elimination is governed by the extent of intestinal absorption of the circulating compounds, which is influenced by the efficacy of intestinal hydrolysis of the conjugates and the relative lipophilicity of the aglycones released.

Introduction

Many drugs and other xenobiotics (see Smith 1973), and certain endogenous compounds (e.g. steroid hormones; see Taylor 1971) are excreted in bile and consequently may undergo an enterohepatic circulation. A variety of xenobiotics are known to undergo extensive enterohepatic circulation in the rat, including the contraceptive steroids norethindrone (norethisterone) and mestranol (Hanasono and Fischer 1974), the synthetic estrogen stilbestrol (Fischer *et al.* 1966), the hypnotic and sedative methaqualone (Polk *et al.* 1974), the DDT metabolite DDA (bis-(*p*-chlorophenyl)acetic acid) (Gingell 1975) and hexachlorophene (Gandolfi and Buhler 1974). All these xenobiotics are excreted in the bile of rats as glucuronides. This recycling of drugs and/or their metabolites may have beneficial or harmful results (see Williams, Millburn and Smith 1965); thus the enterohepatic circulation of the anti-typhoid drug chloramphenicol has toxic consequences in the rat. Chloramphenicol glucuronide, excreted in bile, is hydrolysed to chloramphenicol by gut bacteria. Further metabolism in the intestine results in the reduction of

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chloramphenicol to toxic goitrogenic amines, which are absorbed (Thompson *et al.* 1954). The long duration of action of the xanthine oxidase inhibitor 3-(4-pyrimidinyl)-5-(4-pyridyl)-1,2,4-triazole has been attributed to its enterohepatic circulation, which confines the drug within the major biosynthetic sites for uric acid, i.e. liver and intestine (Duggan *et al.* 1974). The differences between species in the extent of the enterohepatic recycling of indomethacin have been correlated with species sensitivity to intestinal lesions produced by this drug (Duggan *et al.* 1975).

Hepatic extraction of compounds from the blood is usually followed by their metabolism to polar conjugates which are excreted in bile. It is well established that a number of physicochemical and biological factors influence the extent to which organic compounds are eliminated in bile. These factors include molecular weight, molecular structure, polarity, species, sex, and metabolic transformations (see Millburn 1976). Most drugs undergoing biliary elimination are excreted as either glucuronic acid or glutathione conjugates; this is probably because these two conjugations confer a considerable increase in molecular size and introduce a polar carboxylic acid group into the molecule.

Phenolphthalein, which in the rat is conjugated with glucuronic acid and then extensively eliminated in the bile (Millburn, Smith and Williams 1967), was chosen as a model compound to investigate the influence of intestinal metabolism and absorption of biliary-excreted drug conjugates on enterohepatic circulation. These factors are important because they will determine the amount of recycled drug which is *hepatically available*, i.e. available to the liver from the portal circulation, while the extent to which this recycled drug is extracted from the portal blood by the liver will determine its *systemic availability*. A preliminary report of part of this work on phenolphthalein has appeared (Hirom, Millburn and Parker 1976).

The enterohepatic circulation of morphine, lysergic acid diethylamide (LSD) and diphenylacetic acid, all of which are excreted in bile as glucuronides, has also been investigated and compared with that of phenolphthalein.

Materials and methods

Compounds (melting points uncorrected)

Phenolphthalein, m.p. 251°C (Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, UK) and sodium phenolphthalein monoglucuronide (Koch-Light Laboratories Ltd, Colnbrook, Bucks., UK) were purchased. [^{3}H]Phenolphthalein (2·8 μCi/mg; radiochemical purity 98%) was available in this laboratory (see Capel, Millburn and Williams 1974).

Morphine hydrochloride (Macarthy's Laboratories, Romford, Essex, UK), [1(n)-^{3}H]morphine (91 200 μCi/mg; radiochemical purity 98%) (The Radiochemical Centre, Amersham, Bucks., UK) and diphenylacetic acid, m.p. 148°C (Aldrich Chemical Co. Inc., Gillingham, Dorset, UK) were purchased. Morphine 3-sulphate (see Capel *et al.* 1974) and (+)-lysergic acid diethylamide tartrate and (+)-lysergic acid [$^{14}C_1$]diethylamide (^{14}C-LSD; 2·2 and 13·6 μCi/mg; radiochemical purity 97%) (see Barnes 1974) were available in this laboratory. Diphenyl[*carboxy*-^{14}C]acetic acid (3·0 μCi/mg; radiochemical purity 99·5%) and diphenylacetyl glucuronide, m.p. 175°C, were synthesized by Dr P. A. F. Dixon (see Dixon, Caldwell, and Smith 1977).

^{14}C-labelled 13-hydroxy-LSD was prepared as follows. Bile collected from five rats each dosed with ^{14}C-LSD (0·97 mg; sp. activity 2·2 μCi/mg) intraperitoneally, was poured onto a column (300 mm × 10 mm diam.) of Amberlite XAD-2 resin (British Drug Houses), which was then washed with water (100 ml). The column was dried by passing a stream of N_2 through it, the biliary metabolites of ^{14}C-LSD were eluted with methanol (100 ml) and the eluate evaporated to a small volume (2–3 ml). The conc. eluate was applied to preparative t.l.c. plates (silica gel HF_{254}; Merck, from Anderman & Co. Ltd, London, UK; 0·8 mm thick), which were developed using solvent A (table 1). The chromatograms, when viewed under u.v. light, showed two blue fluorescent bands, both containing ^{14}C, at R_F 0·30 and 0·34. These R_F values correspond to those quoted for the glucuronides of 13- and 14-hydroxy-LSD respectively by Siddik (1975). The band at R_F 0·30 was removed from the glass plate into methanol (50 ml), filtered to remove the silica and reduced to dryness *in vacuo*. The residue was taken up in 0·2 M sodium acetate buffer pH 5 (1 ml), a β-glucuronidase preparation (2 ml of Ketodase; see below) added and incubated at 37°C for 24 h; then

Table 1. R_F values of phenolphthalein, morphine, lysergic acid diethylamide (LSD) and diphenylacetic acid and some of their metabolites. T.l.c. was carried out on Kieselgel $60F_{254}$ (0·2 mm thickness) aluminium-backed plates. Solvents (proportions by vol.): A, propan-1-ol–ammonia solution (sp. gr. 0·88) (7 : 3); B, pyridine–pentan-1-ol–water (7 : 7 : 6); C, butan-1-ol–acetic acid–water (4 : 1 : 5); D, butan-1-ol–acetone–acetic acid–M ammonia–water (45 : 15 : 10 : 10 : 20); E, chloroform–methanol–water (5 : 5 : 1); F, chloroform–methanol (4 : 1); G, benzene–acetone–acetic acid (2 : 2 : 1); H, benzene–acetone–acetic acid (6 : 2 : 1). The solvent was allowed to run 150 mm from the origin. Phenolphthalein was made visible as a red-brown spot by spraying the plates with 0·2 M NaOH. Phenolphthalein glucuronide gave this colour after spraying with 0·1 M HCl and heating at 80° for 15 min, followed by spraying with 0·2 M NaOH. Morphine, diphenylacetic acid and their metabolites were located on the plates by their quenching of u.v. light (Hanovia Chromatolite Lamp), whereas LSD and its metabolites gave a blue fluorescence when exposed to u.v. light.

Compound	R_F in solvent: A	B	C	D	E	F	G	H
Phenolphthalein	0·71	0·78	—	—	—	—	—	—
Phenolphthalein glucuronide	0·23	0·44	—	—	—	—	—	—
Morphine	—	—	0·44	0·42	—	—	—	—
Morphine 3-sulphate	—	—	0·23	0·33	—	—	—	—
Morphine 3-glucuronide†	—	—	0·35	0·14	—	—	—	—
Morphine 6-glucuronide†	—	—	0·45	0·22	—	—	—	—
Lysergic acid diethylamide (LSD)	0·77	—	—	—	0·83	0·53	—	—
13-Hydroxy-LSD‡	0·73	—	—	—	0·79	0·39	—	—
13-Hydroxy-LSD glucuronide‡	0·30	—	—	—	0·41	0·00	—	—
14-Hydroxy-LSD‡	0·66	—	—	—	0·78	0·34	—	—
14-Hydroxy-LSD glucuronide‡	0·34	—	—	—	0·49	0·00	—	—
Diphenylacetic acid	—	—	—	—	—	—	0·88	0·61
Diphenylacetylglucuronide	—	—	—	—	—	—	0·10	0·00

† The R_F values for these glucuronides are quoted from Yoshimura *et al.* (1969).
‡ The R_F values for these metabolites of LSD are quoted from Siddik (1975).

methanol (3 ml) was added and the denatured enzyme removed by filtration. The filtrate was evaporated to dryness *in vacuo*, the residue taken into methanol (0·5 ml) and applied to preparative t.l.c. plates (see above), which were developed in solvent F (table 1). The chromatograms showed two fluorescent bands, a small one at the origin (unchanged glucuronide) and a broad band at R_F 0·39, which corresponded to 13-hydroxy-LSD. The latter band was removed from the plate into methanol (50 ml), the silica removed by filtration, and the filtrate taken to dryness *in vacuo*. The residue was taken up into rat bile (10 ml), which was used for intraduodenal infusion studies (see below). T.l.c. of this bile using solvent E (table 1) showed a single ^{14}C-labelled compound which had a blue fluorescence under u.v. light, R_F 0·79, which corresponds to 13-hydroxy-LSD.

Animals

Female Wistar albino rats (150–220 g) from Allington Farms (Porton Down, Salisbury, Wiltshire, UK) or Anglia Laboratory Animals (Alconbury, Huntingdon, Cambs., UK), were fed on 41B nuts supplied by Labsure Animal Diets (Poole, Dorset, UK).

For the separate collection of faeces and urine, the rats were housed in glass metabolism cages ('Metabowls'; Jencons, Hemel Hempstead, Herts., UK). Faeces and urine were collected at daily intervals for four days, during which time the animals were allowed free access to food and water.

Bile duct and duodenal cannulation

Rats were anaesthetized with intraperitoneal pentobarbitone sodium ('Nembutal', Abbott Laboratories Ltd, Queenborough, Kent, UK; 60 mg/kg) and their bile ducts cannulated as described by Abou-El-Makarem *et al.* (1967). For enterohepatic circulation investigations requiring intraduodenal infusion, the bile duct was cannulated as above and then a second cannula, consisting of a length of polypropylene tubing (Portex PP25; internal diameter 0·4 mm, external diam. 0·8 mm; Portland Plastics Ltd, Hythe, Kent), was inserted into the bile duct distally to the first, so that its tip just entered the duodenum, and was then ligatured in place. The abdominal wall was closed around both cannulae with sutures and suture clips. The duodenal cannula was used for the infusion of bile containing radio-labelled compounds (see below).

When bile was collected for periods of time extending beyond the anaesthetic range of pentobarbitone sodium (about 3 h for a single dose of 60 mg/kg), bile-duct-cannulated rats were housed in restraining

cages (see Abou-El-Makarem *et al.* 1967), kept warm with a heating lamp and allowed free access to water. The collection of bile at hourly intervals for up to 24 h was achieved by connecting the bile-duct cannula to a fraction collector (Towers Automatic Fraction Collector, Model A) programmed to change the collecting tubes every hour. Urine was collected in a tray positioned beneath the restraining cage.

Administration of compounds

[^{3}H]Phenolphthalein dissolved in 0·1 ml dimethylsulphoxide (DMSO) was injected i.p. (25 mg/kg) into rats. For intraduodenal administration, [^{3}H]phenolphthalein in 0·1 ml DMSO was mixed with bile (2–4 ml), which was then infused via a duodenal cannula into bile-duct-cannulated rats. Bile (3 h sample) containing [^{3}H]phenolphthalein glucuronide, obtained by i.p. administration of [^{3}H]phenolphthalein to one group of bile-duct-cannulated rats, was infused intraduodenally into a second group of bile-duct-cannulated animals, at 0·64 or 1·28 ml bile per h, using an infusion pump (Palmer Slow Injection Apparatus). In some experiments bile from one rat was drained directly into the duodenum of a second bile-duct-cannulated rat via a linking cannula (Portex PP25). The restraining cage housing the donor rat was positioned approx. 200 mm above the cage housing the recipient to assist the flow of bile from donor to recipient.

[^{3}H]Morphine hydrochloride (5 mg/kg) dissolved in 0·9% NaCl (0·5 ml), ^{14}C-LSD (1 mg/kg) in 0·5% (w/v) aq. (+)-tartaric acid (0·2 ml), and [^{14}C]diphenylacetic acid (25 mg/kg) in propylene glycol (0·2 ml), were injected i.p. into bile-duct-cannulated rats. For the intraduodenal infusion in the compounds, they were dissolved in rat bile (2 ml). Bile containing [^{3}H]morphine 3-glucuronide, or [^{14}C]diphenylacetylglucuronide, was obtained by i.p. administration of the radioactive aglycones to bile-duct-cannulated rats. Bile containing the radioactive metabolites of ^{14}C-LSD (mainly the glucuronides of 13- and 14-hydroxy-LSD) was similarly obtained. The preparation of ^{14}C-labelled 13-hydroxy-LSD dissolved in rat bile is described above. These 3 h bile samples containing radioactive biliary metabolites of [^{3}H]morphine, ^{14}C-LSD or [^{14}C]diphenylacetic acid were then infused intraduodenally into bile-duct-cannulated rats.

Antibiotic treatment

In some experiments rats were treated orally with antibiotics to suppress the intestinal microflora before bile-duct-cannulation and intraduodenal administration of the radio-labelled compounds or their glucuronides. Neomycin sulphate (100 mg; Upjohn Ltd, Crawley, Sussex, UK), tetracycline hydrochloride ('Achromycin'; 50 mg; Lederle Laboratories, Gosport, Hants., UK) and bacitracin (50 mg; Burroughs, Wellcome & Co., London, UK) were suspended in aq. soln. (5% v/v of Tween 80 (polyoxyethylene (20) sorbitan mono-oleate; British Drug Houses)). This antibiotic mixture was administered orally by passing a dosing needle (size 19G; 80 mm long) down the oesophagus of the rat for a distance of about 50 mm. The dosing regime used entailed single daily doses of the antibiotic mixture for 2 days, followed on the third day by a dose 4 h before and another dose 4 h after bile-duct-cannulation and intraduodenal infusion of the rat with a labelled compound.

Radiochemical techniques

^{3}H or ^{14}C in biological fluids was determined by liquid scintillation counting using either a toluene and dioxan-based scintillator for ^{3}H (Capel *et al.* 1974) or Bray's scintillant for ^{14}C (see Bridges, Davies and Williams 1967). In some experiments either ^{3}H- or ^{14}C-labelled samples were counted in a toluene and Triton X–100 (Koch-Light) based scintillator consisting of 2,5-diphenyloxazole (12·5 g) and 1,4-bis(5-phenyloxazol-2-yl)benzene (0·75 g) per 2·5 l toluene, 2 vol. of which were added to 1 vol. of Triton X–100. Samples (0·01–1·0 ml) of urine and bile were counted directly in the appropriate scintillator (10–20 ml). Samples (5–10 g) of intestinal contents and faeces were homogenized in methanol (10 ml) using an 'Ultra-Turrax' homogenizer, and 0·1–1·0 ml of these homogenates were counted in the appropriate scintillator containing a thixotropic gel (Cab-O-Sil; Koch-Light; 5% w/v). Counting efficiencies, determined by the channels ratio method, were 20–28% for samples containing ^{3}H and 70–90% for those containing ^{14}C.

Radiochromatograms on t.l.c. plates (see below) were scanned for ^{3}H or ^{14}C in a Packard radiochromatogram scanner (model 7200). The R_F values of the ^{3}H or ^{14}C peaks were compared with those of authentic reference compounds. The aluminium-backed t.l.c. plates were cut into small pieces (10 mm by 40 mm) and the radioactivity in each piece determined by counting in the above scintillators.

Chromatography

The R_F values of phenolphthalein, morphine, LSD, diphenylacetic acid and some of their metabolites are shown in table 1. Urine and bile samples (0·01–0·1 ml) were chromatographed on silica gel (200 × 40 mm, Kieselgel 60F_{254}, 0·2 mm thick, aluminium-backed t.l.c. plates from E. Merck, Darmstadt, FR Germany). To identify radioactive compounds and their metabolites in faecal homogenates (5 ml), these were shaken with methanol (15 ml) for 30 min, followed by centrifugation at 2000 r.p.m. The methanolic supernatant was reduced to 0·1 ml *in vacuo*, and applied directly to t.l.c. plates. Liquid scintillation counting of samples of the methanol showed that this procedure extracted 70–100% of the radioactivity present.

Identification of glucuronic acid conjugates

To detect glucuronides, a β-glucuronidase preparation (Ketodase; ox liver β-glucuronidase from Warner–Chilcott Laboratories, Eastleigh, Hants, UK) was used as described by Idle, Millburn and Williams (1978).

Measurement of partition ratios

The partition ratios of radio-labelled phenolphthalein, morphine, LSD, 13-hydroxy-LSD, diphenylacetic acid and their respective glucuronides were measured between octan-1-ol and 0·1 M phosphate buffer, pH 7·4, a model system for both biological lipid and aqueous phases (see Leo, Hansch and Elkins 1971, Tute 1971). The radioactive glucuronides were obtained by t.l.c. of bile from rats injected with [^{3}H]phenolphthalein, [^{3}H]morphine, ^{14}C-LSD or [^{14}C]diphenylacetic acid. The radioactive bands corresponding to the glucuronic acid conjugates were removed from the t.l.c. plates into methanol (20 ml), filtered to remove the silica and evaporated to dryness *in vacuo*. The residue containing the ^{3}H- or ^{14}C-labelled conjugate was dissolved in 0·1 M phosphate buffer, pH 7·4 (10 ml). The radioactive aglycones were also dissolved in phosphate buffer (10 ml). These aq. soln. containing the radioactive compounds were then each shaken with octan-1-ol (10 ml) for 30 min, followed by centrifugation at 2000 r.p.m. for 10 min to break up any emulsions formed. Samples (0·05 ml) of both the upper organic layer and the aq. layer were counted for ^{3}H or ^{14}C. All determinations were carried out in duplicate.

Results

The excretion of [^{3}H]phenolphthalein in faeces and urine of rats

Following administration of [^{3}H]phenolphthalein (25 mg/kg, i.p.) to rats, 96% dose was recovered in four days, 86% in the faeces and 10% in the urine, mostly during the first 48 h (table 2).

T.l.c. and radiochromatogram scanning of a methanol extract from faeces (see *Materials and methods*) showed two ^{3}H peaks, one chromatographically identical to phenolphthalein and the other to phenolphthalein glucuronide. Phenolphthalein accounted for the major proportion of the radioactivity in faeces; for example, on day 1, 83% of the ^{3}H in faeces was as phenolphthalein and 17% as phenolphthalein glucuronide. By contrast, t.l.c. of urine samples showed only a single metabolite, corresponding to phenolphthalein glucuronide. The identity of phenolphthalein glucuronide present in both faeces and urine was confirmed by β-glucuronidase hydrolysis, which completely hydrolysed the conjugate to give a single ^{3}H peak identical on t.l.c. with phenolphthalein.

Table 2. The excretion of [^{3}H]phenolphthalein in faeces and urine of rats. [^{3}H]Phenolphthalein dissolved in 0·1 ml DMSO (10 μCi/animal) was injected i.p. (25 mg/kg) into female Wistar albino rats. Faeces and urine were collected daily for four days. Values given are the mean of three animals, together with S.E.M.

Day	Radioactivity excreted in: (% dose)	
	Faeces	Urine
1	20 ± 9·5	6·0 ± 0·5
2	49 ± 4·5	2·6 ± 0·8
3	9·8 ± 3·1	0·8 ± 0·2
4	7·3 ± 3·0	0·6 ± 0·2
Total (days 1–4)	86 ± 4	10 ± 1·0

Biliary excretion of [^{3}H]phenolphthalein

Following the i.p. injection of [^{3}H]phenolphthalein (25 mg/kg), bile-duct-cannulated rats excreted 95% dose in the bile and only 0·2% in the urine in 24 h (table 3). T.l.c. and radiochromatogram scanning of bile samples showed a single

Table 3. Biliary excretion of [^{3}H]phenolphthalein, [^{3}H]morphine, [^{14}C]lysergic acid diethylamide (LSD) and [^{14}C]diphenylacetic acid. [^{3}H]phenolphthalein in 0·1 ml DMSO, [^{3}H]morphine hydrochloride in 0·5 ml of 0·9% NaCl, [^{14}C]lysergic acid diethylamide in 0·2 ml of 0·5% (w/v) aq. (+)-tartaric acid and [^{14}C]diphenylacetic acid in 0·2 ml propylene glycol were injected into bile-duct-cannulated female Wistar albino rats. Bile and urine were collected at the time intervals shown for 24 h. Values given are means of three animals, together with S.E.M.

	Radioactivity excreted (% dose)			
Time (h)	[^{3}H]Phenolphthalein (25 mg/kg)	[^{3}H]Morphine (5 mg/kg)	^{14}C-LSD (1 mg/kg)	[^{14}C]Diphenylacetic acid (25 mg/kg)
Bile				
1	60 ± 1·7	31 ± 2·9	56 ± 3·9	27 ± 5·9
2	21 ± 2·0	8·6 ± 3·1	14 ± 3·8	21 ± 2·1
3	8·5 ± 1·8	5·3 ± 2·2	4·6 ± 5·2	8·5 ± 1·3
3–24	5·5 ± 2·5	3·6 ± 2·7	4·2 ± 3·0	12 ± 5·4
Urine				
0–24	0·2 ± 0·01	27 ± 3·9	5·7 ± 0·1	9·8 ± 6·8

metabolite chromatographically identical to phenolphthalein glucuronide, which gave phenolphthalein on β-glucuronidase hydrolysis.

Table 3 shows that phenolphthalein is rapidly excreted in bile; about 90% dose in 3 h. However, it takes four days for this amount to appear in faeces (table 2), suggesting that extensive intestinal absorption and enterohepatic recycling of phenolphthalein occurs in the rat. Furthermore, the excretion of phenolphthalein in the 24 h urine of intact rats (6% dose; table 2) is substantially higher than that of bile-duct-cannulated animals (0·2%; table 3), which also suggests an enterohepatic circulation of phenolphthalein.

Enterohepatic circulation of [^{3}H]phenolphthalein

Enterohepatic recycling of phenolphthalein in the rat was demonstrated by 'linked' experiments where bile from one rat, given 25 mg/kg [^{3}H]phenolphthalein i.p., was passed directly via a cannula from its bile duct into the duodenum of a second rat, from which urine, faeces and bile were collected. In three such experiments 54 ± 4% of the dose administered to the first rat was recovered in the bile of the second rat, with only 0·8 ± 0·1% in the urine and 5·4 ± 3·7% in the faeces.

The fate of phenolphthalein and its glucuronide in the intestine

To determine whether phenolphthalein glucuronide, after biliary excretion, is absorbed as such from the intestine or has first to be hydrolysed to phenolphthalein, the fate of the free drug and its glucuronic acid conjugate in the intestine was investigated. Bile containing [^{3}H]phenolphthalein or its glucuronide was infused intraduodenally into bile-duct-cannulated rats at a rate of 0·64 ml/h for up to 3 h. This was the closest possible rate obtainable with the infusion pump to the rate of bile flow in bile-duct-cannulated rats, which in our experiments was 0·7 ± 0·2 ml/h.

Following the intraduodenal infusion of [^{3}H]phenolphthalein there was rapid biliary excretion of radioactivity (60% dose in 4 h (see figure 1 (*a*)). In contrast, after infusion of bile containing [^{3}H]phenolphthalein glucuronide obtained from rats injected with [^{3}H]phenolphthalein, there is a lag period of some 4 h (only 20% dose is eliminated in the bile in this time) before a similar rate of excretion occurs. Bile

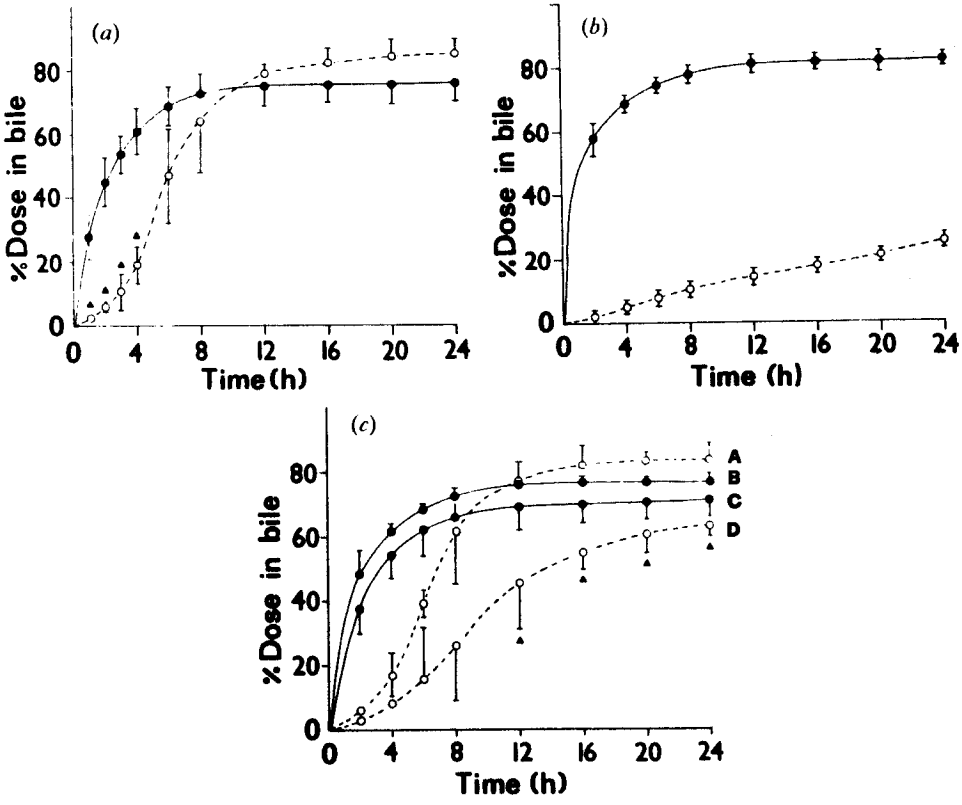

Figure 1. Biliary excretion of radioactivity after intraduodenal infusion of bile containing [^{3}H]phenolphthalein or [^{3}H]phenolphthalein glucuronide into bile-duct-cannulated rats. Bile containing [^{3}H]phenolphthalein (●—●) or [^{3}H]phenolphthalein glucuronide (○---○) was infused intraduodenally over a 3 h period at an infusion rate of 0·64 ml/h ((*a*), (*b*) and (*c*)—curves A and B) giving a dose of 79 μmol/kg or 1·28 ml/h ((*c*)—curves C and D) giving a dose of 158 μmol/kg. The vertical bars represent S.E.M. ($n=3$).
(*a*) Without antibiotic treatment. ▲ These values are significantly ($P<0·05$) lower than the corresponding values for phenolphthalein.
(*b*) After 3 days' treatment with antibiotics (see text).
(*c*) Without antibiotic treatment, but with different infusion rates (see above). ▲ These values for curve D are significantly ($P<0·02$) lower than the corresponding values for curve A.

collected from animals infused with either radioactive phenolphthalein or phenolphthalein glucuronide contained only [^{3}H]phenolphthalein glucuronide, when analysed by t.l.c.

These results show that in the rat phenolphthalein is readily absorbed from the intestine, but phenolphthalein glucuronide is absorbed more slowly. This suggests that the glucuronide may require hydrolysis to the aglycone before significant absorption occurs, as does stilbestrol mono-glucuronide (Fischer *et al.* 1966).

The role of the gut flora in the enterohepatic recycling of phenolphthalein

The gut bacteria make a major contribution to the β-glucuronidase activity found in intestinal contents (see Scheline 1973) and, therefore, the effect of antibiotic treatment on the fate of phenolphthalein and its glucuronide in the intestine was investigated.

Rats were treated daily for three days with antibiotics (neomycin, tetracycline and bacitracin) to suppress the intestinal microflora. This treatment did not inhibit the absorption of free phenolphthalein (figure 1 (*b*)). However, following the duodenal infusion of bile containing [^{3}H]phenolphthalein glucuronide, the biliary excretion of radioactivity (22% dose in 24 h) (figure 1 (*b*)) was much lower than in untreated rats (85% dose) (figure 1 (*a*)). In the antibiotic-treated rats, the remainder of the dose was recovered mostly in the intestinal contents (67 ± 3%) with only small amounts ($0{\cdot}3 \pm 0{\cdot}1$%) in the urine.

Thus the antibiotic treatment markedly decreases the absorption of radioactive material from the intestine following intraduodenal administration of [^{3}H]phenolphthalein glucuronide. Since the antibiotic treatment did not decrease intestinal absorption of free phenolphthalein, hydrolysis of phenolphthalein glucuronide by the gut microflora β-glucuronidase must be important in the enterohepatic circulation of phenolphthalein.

The capacity of the gut flora to hydrolyse phenolphthalein glucuronide may be rate-limiting in enterohepatic circulation. Phenolphthalein injected intraperitoneally into rats is rapidly excreted in bile in a conjugated form (table 3) and the rate at which the glucuronide enters the intestine can determine the extent of enterohepatic circulation. Thus, when bile containing [^{3}H]phenolphthalein glucuronide was infused intraduodenally into bile-duct-cannulated rats at a rate of 1·28 ml/h, the amount recovered in bile in 24 h (62% dose; curve D, figure 1 (*c*)) was less than that recovered (85%) when the same bile was infused at a rate of 0·64 ml/h (curve A, figure 1 (*c*)). This difference may be due to the saturation of the intestinal β-glucuronidase, since there was no significant difference between the recoveries of ^{3}H in the bile of rats infused intraduodenally with bile containing [^{3}H]phenolphthalein at either 0·64 or 1·28 ml/h (curves B and C, figure 1 (*c*)).

Biliary excretion of [^{3}*H*]*morphine,* [^{14}*C*]*lysergic acid diethylamide* (*LSD*) *and* [^{14}*C*]*diphenylacetic acid*

Morphine. Following the intraperitoneal administration of [^{3}H]morphine (5 mg/kg) to bile-duct-cannulated rats, approx. 50% dose was recovered in the bile in 24 h and about 25% in the urine (table 3). T.l.c. and radiochromatogram scanning of bile samples showed one ^{3}H peak at R_F 0·35 (solvent C) and 0·14 (solvent D; table 1). These R_F values correspond to those for morphine 3-glucuronide (Yoshimura, Oguri and Tsukamoto 1969). The radioactive metabolite in rat bile was hydrolysed to morphine by β-glucuronidase. T.l.c. of urine samples also showed the presence of morphine 3-glucuronide. The recovery of morphine in the bile was similar to that reported by March and Elliott (1954; 63% dose) and Walsh and Levine (1975; 49% dose).

Lysergic acid diethylamide (*LSD*). Nearly 80% of a dose (1 mg/kg i.p.) of ^{14}C-LSD was excreted in the bile of rats in 24 h with only about 5% in the urine (table 3). Two major metabolites (R_F 0·41 and 0·49, solvent E, table 1) were found in bile. These R_F values correspond to those for the glucuronides of 13- and 14-hydroxy-LSD respectively (Siddik 1975; see also Siddik *et al.* 1975). These two glucuronides together accounted for 83% of the radioactivity in bile; the remainder was accounted for by two unknown metabolites, present in approx. equal amounts (see also Siddik *et al.* 1975). These unknown metabolites were not hydrolysed by β-glucuronidase and were not examined further.

Diphenylacetic acid. Some 70% dose (25 mg/kg, i.p.) of [^{14}C]diphenylacetic acid was excreted in the bile of rats in 24 h with about 10% in the urine (table 3). T.l.c. of bile samples showed two ^{14}C peaks. The larger peak, R_F 0·10 (solvent G; table 1), corresponded to diphenylacetylglucuronide (87% of the ^{14}C in bile) and the smaller, R_F 0·88 (solvent G), to unchanged diphenylacetic acid (13%). The identity of the glucuronic acid conjugate was confirmed by β-glucuronidase hydrolysis, which gave diphenylacetic acid. Dixon *et al.* (1977) have also reported that diphenylacetic acid is conjugated with glucuronic acid and extensively excreted in the bile of rats.

Morphine, LSD and diphenylacetic acid are, therefore, all substantially and rapidly excreted in the bile of rats as glucuronic acid conjugates (table 3). Thus, in 2 h following injection of morphine or LSD, >80% of the total biliary excretion has occurred; for diphenylacetic acid the figure is 70%.

Enterohepatic circulation of [^{3}H]morphine, ^{14}C-LSD and [^{14}C]diphenylacetic acid

Morphine. Following the intraduodenal infusion of [^{3}H]morphine into bile-duct-cannulated rats, there is a rapid biliary excretion of radioactivity (35% dose in 4 h, 40% in 8 h) (figure 2(*a*)). The biliary excretion of ^{3}H following intraduodenal infusion of [^{3}H]morphine 3-glucuronide is, however, much slower (< 5% dose in 4 h and only 10% in 8 h).

Although the rate of excretion of radioactivity is different, the total recovery of ^{3}H in 24 h is the same for both morphine (40 ± 1% dose in bile, 25 ± 5% in urine, 14 ± 5% in intestinal contents) and its glucuronide (bile 41 ± 5%, urine 20 ± 4% and gut contents 11 ± 3%). Bile and urine from animals infused with either morphine or its glucuronide contained only morphine 3-glucuronide, by t.l.c. analysis. Thus, in these experiments 60–65% of the radioactivity was absorbed from the intestine in 24 h and about two thirds of this underwent recycling. Consequently, there was a substantial loss of material (one third of that absorbed) from the enterohepatic circulation to the urine. This may explain the apparent difference in urinary

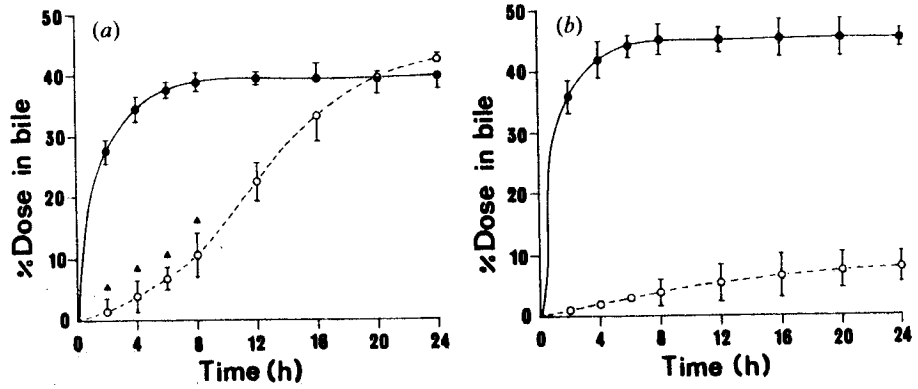

Figure 2. Biliary excretion of radioactivity after the intraduodenal infusion of bile containing [^{3}H]morphine or [^{3}H]morphine 3-glucuronide into bile-duct-cannulated rats. Bile containing [^{3}H]morphine (●—●) or [^{3}H]morphine 3-glucuronide (○- - -○) was infused intraduodenally over a 3 h period at an infusion rate of 0·64 ml/h giving a dose of 7·9 μmol/kg. The vertical bars represent S.E.M. (n=3).

(*a*) Without antibiotic treatment. ▲ These values are significantly (P<0·01) lower than the corresponding values for morphine.

(*b*) After 3 days' treatment with antibiotics (see text).

excretion of a dose of morphine in bile-duct-cannulated rats (27% dose in 24 h (table 3)) compared with intact rats (52% dose in the urine in 24 h and 24% in the faeces (March and Elliott 1954)).

Suppressing the activity of the gut micro-organisms with antibiotics did not inhibit the absorption of free [^{3}H]morphine (figure 2 (*b*)) (recovery of ^{3}H in bile 44 $\pm$3%; urine 21$\pm$5%, intestinal contents 20$\pm$5%). However, after intraduodenal infusion of [^{3}H]morphine 3-glucuronide into animals treated with antibiotics, most (51$\pm$6%) of the ^{3}H remained in the intestinal contents, with 12$\pm$3% in the urine and only 8·6$\pm$3·0% in the bile (figure 2 (*b*)).

LSD. This drug is excreted in the bile of rats mainly as glucuronic acid conjugates of 13- and 14-hydroxy-LSD. When bile from rats previously injected with ^{14}C-LSD was infused intraduodenally into bile-duct-cannulated rats, only about half of the radioactivity was absorbed (35% in 24 h); 28$\pm$4% was in the bile (figure 3) and 6·7$\pm$3·2% in urine. The faeces are the major final excretory route for LSD; intact rats excrete 72% of an injected dose in faeces in 48 h, and 15% in the urine (Siddik *et al.* 1975). Bile-duct-cannulated rats excrete 6% in the urine in 24 h (table 3), and the difference (9%) from that excreted by the intact rat is similar to the amount (6·7% dose) in the urine after intraduodenal administration of ^{14}C-LSD metabolites to bile-duct-cannulated rats. This shows that only small amounts of LSD or its metabolites escape from the enterohepatic circulation for renal excretion.

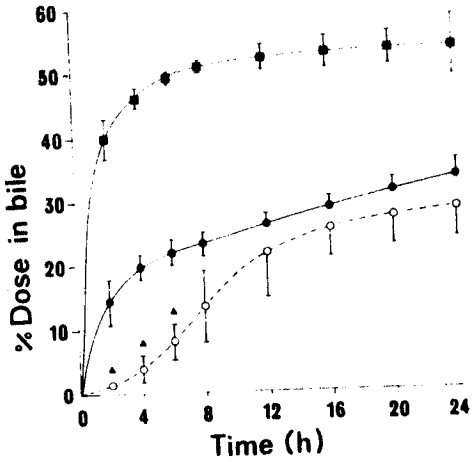

Figure 3. Biliary excretion of radioactivity after the intraduodenal infusion of bile containing either [^{14}C]-lysergic acid diethylamide, ^{14}C-13-hydroxy-LSD, or the biliary metabolites of ^{14}C-LSD. Bile containing ^{14}C-LSD (■—■), ^{14}C-13-hydroxy-LSD (●—●) or the biliary metabolites of ^{14}C-LSD (○- - -○) was infused intraduodenally over a 3 h period at an infusion rate of 0·64 ml/h giving a dose equivalent to 2·3 μmol of LSD/kg. The vertical bars represent S.E.M. (n=3).
▲ These values are significantly (P<0·01) lower than the corresponding values for 13-hydroxy LSD.

Bile containing ^{14}C-13-hydroxy-LSD, the aglycone of a major biliary metabolite of LSD, was also infused intraduodenally into bile-duct-cannulated rats. The rate of biliary excretion of ^{14}C was greater than when conjugated 13- and 14-hydroxy-LSD was infused, although the total excretion of ^{14}C in bile was similar in both cases (figure 3). Bile from rats given ^{14}C-13-hydroxy-LSD contained mainly 13-hydroxy-LSD glucuronide (88% of biliary ^{14}C).

Diphenylacetic acid. [^{14}C]Diphenylacetic acid was infused intraduodenally into bile-duct-cannulated rats and 75 ± 5% of the dose was excreted in bile in 24 h, 8·7 ± 1·8% in urine, and 14 ± 6% in the gut contents. Bile from rats injected with [^{14}C]diphenylacetic acid, which contained mainly [^{14}C]diphenylacetylglucuronide, was infused intraduodenally into bile-duct cannulated rats. The recovery of ^{14}C in 24 h was: bile 66 ± 4%, urine 7·3 ± 2·1%, intestinal contents 15 ± 4%. The total biliary excretion of ^{14}C is, therefore, similar on infusing [^{14}C]diphenylacetic acid or its glucuronic acid conjugate, but the rate of excretion of biliary ^{14}C is faster in the former case (figure 4 (*a*)).

Antibiotic treatment, as for phenolphthalein and morphine, did not inhibit the absorption and subsequent biliary excretion of [^{14}C]diphenylacetic acid, but did inhibit that of [^{14}C]diphenylacetylglucuronide, the major component (85–90%) of infused bile (figure 4 (*b*)). In antibiotic-pretreated animals, only 20% of the infused biliary metabolites were absorbed and re-excreted in the bile in 24 h. Since unconjugated diphenylacetic acid comprised 10–15% of the infused material, at least half of the amount re-excreted could originate from this source.

Intact rats excrete 48% of an injected dose of [^{14}C]diphenylacetic acid in the urine in 48 h (Dixon *et al.* 1977). Bile-duct-cannulated rats, however, eliminate 69% dose in the bile in 24 h, but only about 10% in the urine (table 3). This difference between intact and bile-duct-cannulated animals implies that a substantial fraction of the dose escapes from the enterohepatic circulation and is eliminated in the urine.

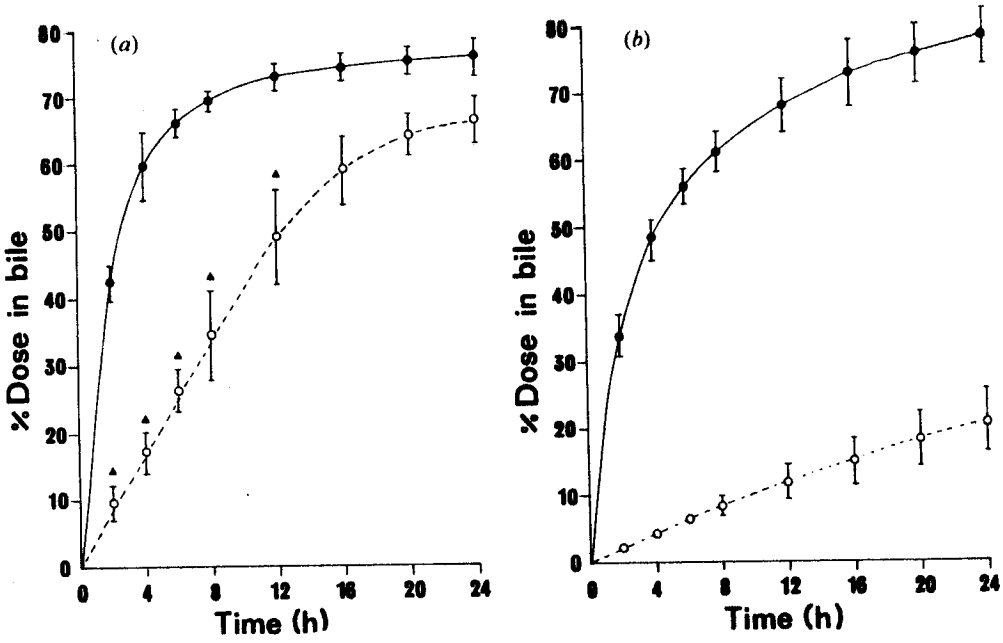

Figure 4. Biliary excretion of radioactivity after the intraduodenal infusion of bile containing [^{14}C]diphenylacetic acid or [^{14}C]diphenylacetylglucuronide. Bile containing [^{14}C]diphenylacetic acid (●—●) or [^{14}C]diphenylacetylglucuronide (○- - -○) was infused intraduodenally over a 3 h period at an infusion rate of 0·64 ml/h giving a dose of 66·6 μmol/kg. The vertical bars represent S.E.M. ($n = 3$).
(*a*) Without antibiotic treatment. ▲ These values are significantly ($P < 0·02$) lower than the corresponding values for diphenylacetic acid.
(*b*) After 3 days' treatment with antibiotics (see text).

Partition ratios

Relative lipid-solubilities of compounds were determined by measuring their partition ratios (P) between octan-1-ol and 0·1 M phosphate buffer, pH 7·4 (table 4). Phenolphthalein has a considerably higher P value (3·64) than phenolphthalein glucuronide (0·19), and is more rapidly absorbed from the intestine during enterohepatic circulation (figure 1). Morphine, 13-hydroxy-LSD and diphenylacetic acid also have higher P values (i.e. are more lipid-soluble) than their respective glucuronides and, like phenolphthalein, these aglycones are more readily absorbed from the gut than their conjugates (figures 2–4).

Table 4. Partition ratios of phenolphthalein, morphine, LSD, diphenylacetic acid and some of their metabolites between octan-1-ol and 0·1 M phosphate buffer, pH 7·4.
The partition ratios (P) for octan-1-ol to buffer were measured as described in the text.

Compound	P
Phenolphthalein	3·64
Phenolphthalein glucuronide	0·19
Morphine	0·81
Morphine 3-glucuronide	0·11
LSD	7·07
13-Hydroxy-LSD	0·46
13-Hydroxy-LSD glucuronide	0·05
Diphenylacetic acid	7·93
Diphenylacetyl glucuronide	0·38

Intraduodenally infused aglycones with a high P value, e.g. phenolphthalein ($P=3·64$) and diphenylacetic acid ($P=7·93$), show greater intestinal absorption and re-excretion, with $72\pm5\%$ and $76\pm6\%$ of [^{3}H]phenolphthalein (see curves B and C, figure 1 (*c*)) appearing in bile and $<1·0\%$ in urine, and $76\pm3\%$ of [^{14}C]diphenylacetic acid in bile (figure 4 (*a*)) and $8·7\pm1·8\%$ in urine. This compares with lower values for [^{3}H]morphine ($P=0·81$) of $40\pm1\%$ in bile (figure 2 (*a*)) and $25\pm5\%$ in urine, and only $34\pm3\%$ of 13-hydroxy-LSD ($P=0·46$) in bile and $5·0\pm3·5\%$ in urine.

Discussion

Intestinal hydrolysis of glucuronides during enterohepatic circulation

Comparing the enterohepatic circulation of phenolphthalein, morphine, LSD and diphenylacetic acid, all of which are excreted in the bile as glucuronic acid conjugates, the following similarities are apparent. The glucuronides are slowly absorbed from the intestine and are hydrolysed to their respective aglycones, which being more lipid-soluble are then rapidly absorbed. The β-glucuronidase of the gut microflora is largely responsible for the hydrolysis of these conjugates, since antibiotic treatment markedly decreases the absorption of radioactive material from the intestine following intraduodenal administration of the glucuronides of [^{3}H]phenolphthalein, [^{3}H]morphine and [^{14}C]diphenylacetic acid. Walsh and Levine (1975) have also shown that gut bacterial hydrolysis of morphine glucuronide is a prerequisite for enterohepatic circulation of the drug in the rat, and that this hydrolysis can be inhibited by treating the animals with the antibiotic lincomycin. Similarly, antibiotic-treatment decreases the enterohepatic recycling of the synthetic estrogen stilbestrol (Clark *et al.* 1969, Fischer, Kent and Weissinger 1973) and

of the contraceptive steroid mestranol (Brewster, Jones and Symons 1976). The naturally occurring steroid, pregnanolone, is biliary-excreted as a glucuronide in the rat, and undergoes enterohepatic circulation during which it is hydrolysed in the intestine to the free steroid before absorption (Long and Soyka 1975).

Since antibiotic-treatment did not completely abolish the enterohepatic circulation of administered phenolphthalein glucuronide or morphine 3-glucuronide (figures 1 and 2), small amounts of these conjugates may be absorbed unchanged from the intestine. Alternatively hydrolysis could be catalysed by β-glucuronidase of biliary or intestinal mucosal origin (Cooper, Eakins and Slater 1976, Marselos, Dutton and Hänninen 1975).

Lipid-solubility and intestinal absorption during enterohepatic circulation

Studies of drug absorption have shown that lipid-soluble, unionized molecules readily penetrate the intestinal epithelium (see Schanker 1960), and it has been suggested that there is an optimal partition coefficient (octanol/buffer) for intestinal absorption of xenobiotics (Houston, Upshall and Bridges 1974).

The data presented here show that the gut bacterial β-glucuronidase hydrolysis of the conjugates during enterohepatic circulation changes the physicochemical properties of the circulating molecules, making the molecules more lipid-soluble. This favours intestinal absorption and is crucial in enterohepatic circulation, since the conjugates themselves are poorly absorbed, as shown by our studies in antibiotic-treated animals. The relative extents of absorption and re-excretion of the aglycones may also be correlated with lipid-solubility. This is illustrated by comparing compounds with high and low octan-1-ol/phosphate buffer partition ratios (P) (see *Results* section) or by comparing 13-hydroxy LSD ($P=0{\cdot}46$) with LSD itself ($P=7{\cdot}07$). Following intraduodenal infusion of ^{14}C-13-hydroxy LSD, less was absorbed and re-excreted in the bile and urine ($33\pm3\%$ and $5{\cdot}0\pm3{\cdot}5\%$ respectively) than was the case with ^{14}C-LSD itself ($54\pm4\%$ in bile and $6{\cdot}4\pm1{\cdot}6\%$ in urine) (see figure 3). Nearly half the dose of 13-hydroxy-LSD ($48\pm6\%$) was unabsorbed and remained in the intestinal contents compared with only $25\pm2{\cdot}7\%$ of the intraduodenal dose of LSD. LSD is hydroxylated and conjugated with glucuronic acid *in vivo* and then excreted in bile. Bacterial β-glucuronidase hydrolysis releases 13-hydroxy-LSD into the intestine, but because this compound has a relatively low lipid-solubility, it is poorly absorbed.

Pharmacological implications of enterohepatic circulation

One question to be asked in connection with the enterohepatic circulation of any drug is: "Does this recycling result in the presence in the peripheral circulation of significant amounts of the pharmacologically active form(s) of the drug?"

The depression of locomotor activity caused by phenobarbital or progesterone, which are excreted in the bile as glucuronides, is shortened in rats pretreated with saccharo-1,4-lactone, a potent inhibitor of β-glucuronidase (Marselos *et al.* 1975). It was suggested that this inhibition of hydrolysis of biliary-excreted glucuronic acid conjugates suppressed the enterohepatic recycling of their pharmacologically active aglycones. However, in the case of phenolphthalein, enterohepatic circulation results in the systemic bioavailability of phenolphthalein glucuronide, not of the unconjugated drug. For in intact, but not in bile-duct-cannulated, rats there is a second peak in blood radioactivity, which occurs 5–6 h after intravenous administration of [^{3}H]phenolphthalein (Hirom *et al.* 1976, Colburn *et al.* 1979), and coincides

with the absorption of [^{3}H]phenolphthalein from the intestine following the bacterial β-glucuronidase hydrolysis of the biliary-excreted [^{3}H]phenolphthalein glucuronide (figure 1 (*a*)). Although portal blood of intact rats contains both free [^{3}H]phenolphthalein and its glucuronide, all radioactivity in the systemic circulation is present as the conjugate (Colburn *et al.* 1979). Therefore, the first pass of phenolphthalein through the intestinal epithelium and liver results in the total metabolism of the phenol to its glucuronide, most occurring in the liver (Parker 1977).

A further factor that will influence the amount of unmetabolized drug reaching the peripheral circulation is the rate of intestinal absorption of the free drug released by bacterial hydrolysis of the biliary conjugate(s). β-Glucuronidase is found mainly in the caecum and colon (Marsh, Alexander and Levvy 1952) and for both stilbestrol (Fischer *et al.* 1973) and morphine (Walsh and Levine 1975) the major site of release from their conjugates is the caecum, but here the unconjugated drugs are relatively slowly absorbed. This slow absorption of the free drugs is unlikely to saturate either their hepatic extraction from the portal blood or the first-pass metabolic capacity of the liver (and/or intestinal wall). Thus, in enterohepatic circulation of stilbestrol, the biliary conjugate, stilbestrol monoglucuronide, is hydrolysed in the intestine releasing stilbestrol, which is reabsorbed. During its passage across the gut wall, the drug is partly reconjugated with glucuronic acid and about 50% of the stilbestrol content of the portal blood is present as the glucuronide (Fischer and Millburn 1970). The drug and its conjugate are extracted from the portal blood by the liver, the remainder of the free stilbestrol conjugated, and the conjugate re-excreted in bile. This hepatic extraction is so efficient that the blood levels of radioactivity following administration of [^{14}C]stilbestrol are not any different in intact rats from those in bile-duct-cannulated animals, in which the enterohepatic circulation has been interrupted (see Smith and Millburn 1975).

Persistence of compounds due to enterohepatic circulation

Phenolphthalein undergoes extensive enterohepatic circulation, and is well absorbed from the rat intestine. Only small amounts are lost from the enterohepatic circulation by urinary excretion, and its final route of elimination is the faeces. Diphenylacetic acid likewise undergoes considerable enterohepatic circulation, but about half the dose is excreted in the urine in 24 h. Both LSD and morphine show more limited enterohepatic circulation which, in the case of LSD, may be because the hydroxylated metabolites released by intestinal hydrolysis of their glucuronides have low lipid-solubility, and are therefore poorly absorbed so that the drug is eliminated mainly in the faeces. Morphine, on the other hand, is well absorbed from the intestine but suffers substantial (about one third) loss from the enterohepatic circulation to the urine, so that the morphine remaining in the enterohepatic circulation rapidly decreases.

The influence of the faecal elimination rate on the blood concentration/time profile of compounds undergoing enterohepatic circulation has been illustrated in the case of phenolphthalein (Colburn *et al.* 1979). Similarly, the urinary excretion rate will obviously influence the persistence of a compound in the blood. If a drug were to undergo enterohepatic circulation so efficiently that losses to the faeces and urine were almost negligible, then it would persist in the body for a very long period

of time. Iophenoxic acid, a radio-opaque medium for oral cholecystography, illustrates this situation and has a plasma half-life of at least 2·5 years in the dog (Mudge *et al.* 1971).

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