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THE BILIARY EXCRETION AND ENTEROHEPATIC CIRCULATION OF BENZO (*a*)PYRENE AND ITS METABOLITES IN THE RAT

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Abstract—The enterohepatic circulation of benzo(*a*)pyrene (BP) has been investigated in the rat with a view to determining the availability of potentially toxic metabolities to tissues within this cycle. Some 60% of the dose of [^{14}C]-BP (3 μmoles kg^{-1}, i.v.) is excreted in bile in 6 hr, with less than 3% in urine. The biliary metabolities are mainly polar conjugates; only 8% of the ^{14}C in 2 hr bile samples can be directly extracted into ethyl acetate. However, following hydrolysis by β-glucuronidase some 40% of the ^{14}C is extractable at pH 7. The extract consisted of polar metabolites (polyhydroxylated and/or conjugated; 37.5%), BP 4,5-diol (16.8%), BP 3,6-quinone (5.9%), 9-hydroxy BP (5.4%) and 3-hydroxy BP (5.3%) as indicated by co-chromatography with authentic standards on reversed phase HPLC, together with several unidentified metabolites. The proximate carcinogen BP 7,8-diol was not detected. Biliary metabolites of [^{14}C]-BP undergo enterohepatic recirculation in the rat; following the intraduodenal infusion of bile containing metabolites of [^{14}C]-BP into bile duct cannulated rats, approximately 20% of the dose is absorbed and excreted in bile in 30 hr, with only 1% in urine. The pattern of metabolites in this bile is very similar to that in bile from rats administered [^{14}C]-BP i.v. Following a single i.v. dose of [^{14}C]-BP (3 μmoles kg^{-1}) to rats with re-entrant bile duct cannulae, which allowed intermittent collection of bile over a period of several days with minimal interference to the enterohepatic circulation, the proximate carcinogen BP 7,8-diol was detected in recirculating bile. Biliary metabolites of BP, which have recently been shown to be mutagenic, can thus traverse the intestine to undergo enterohepatic circulation in the rat.

Benzo(*a*)pyrene (BP)† is a widespread environmental contaminant which can be enzymically converted to mutagenic, cytotoxic and carcinogenic metabolites [1–7]. Microsomal monooxygenases can oxidise BP to produce various phenols, quinones and oxides [8–10]. The oxides may be directly conjugated with glutathione [11], or may undergo hydration to dihydrodiols by epoxide hydrolase (E.C. 4.2.1.63) [12] or further oxidation [13]. In particular the 7,8-diol may be further metabolised to 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene, which covalently binds to cellular DNA and has been shown to be a potent mutagen and carcinogen [4, 14, 15]. Phenolic metabolites, though substrates for UDP-glucuronyltransferase (E.C. 2.4.1.17) [16] and for sulphotransferase (E.C. 2.8.2.1) [17], can also be mutagenic [18] or further metabolised to mutagenic reactive metabolites [14, 19, 20]. 9-OHBP can be metabolised to a form which binds to DNA [21, 22] but it is not known if this reaction can lead to the initiation of carcinogenesis. The metabolism of BP seems qualitatively similar in the various mammalian systems studied. The major ethyl acetate-soluble metabolites formed co-chromatograph with various tetrols and triols, the 4,5-, 7,8- and 9,10-diols, various quinones and 3- and 9-OHBP. [23–28].

The metabolism of BP *in vivo* has, however, received relatively little attention since the early work of Kotin *et al.* [29, 30]. Many of the conjugated metabolites of BP are excreted via the bile in the rat [29] and also in the rabbit [31], a species more similar to man with respect to the biliary excretion of foreign compounds [32]. Recently, biliary metabolites of benzo(*a*)pyrene have been shown to be mutagenic to tester strains of *Salmonella typhimurium* [33]. Furthermore, BP conjugates in rat bile have been shown to be hydrolysed by rat and human intestinal microflora [34] and the products could then be further metabolised by intestinal mucosal cells [35, 36]. These processes may influence the availability of potentially mutagenic and carcinogenic metabolites to intestinal cells and will determine the amount and nature of compounds taken up by the portal blood and carried to the liver. The efficiency of hepatic extraction will, in turn, determine the extent to which such compounds escape from the portal blood to the systemic circulation. Enterohepatic circulation may thus lead to the availability of benzo(*a*)pyrene metabolites to a number of tissues of varying susceptibility to BP-induced carcinogenesis. In this paper we describe studies on this enterohepatic circulation in the rat. Some of this work has been presented in preliminary form elsewhere [37].

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† Abbreviations used: BP: benzo(*a*)pyrene; 3-OHBP and 9-OHBP: 3- and 9-hydroxy BP respectively. BP 9,10-diol, BP 4,5-diol and BP 7,8-diol: 9,10-, 4,5- and 7,8-dihydro-dihydroxy BP; HPLC: high performance liquid chromatography.

MATERIALS AND METHODS

Chemicals and biochemicals. [7,10-^{14}C]-BP was obtained from the Radiochemical Centre, Amersham, Bucks., and was diluted with unlabelled BP (Sigma Chemical Co. Ltd., Poole, Dorset) to give the appropriate specific radioactivity. Authentic standards of metabolites (indicated in Fig. 1) were obtained through the National Cancer Institute, Carcinogenesis Research Program, Bethesda, MD., U.S.A.

β-Glucuronidase (Ketodase®) was obtained from W. R. Warner and Co. Ltd., Eastleigh, Hants., and aryl sulphatase (type H-1, from *Helix pomatia*) from Sigma. Standard laboratory reagents were of AnalaR grade or of the highest purity available. HPLC grade methanol was obtained from Rathburn Chemicals Ltd., Walkerburn, Peebleshire, Scotland. The water used for HPLC was AnalaR grade (BDH Chemicals Ltd., Poole, Dorset).

Animals and treatment. Male Wistar rats (180–250 g) (Lions Lab., Ringwood, Hants.) were used. Water and Labsure diet 41B-modified (RHM, Poole, Dorset) were provided *ad libitum*.

Bile duct cannulations were performed on rats anaesthetised with sodium pentobarbitone (Sagatal®; May and Baker Ltd., Dagenham, Essex, 50 mg kg^{-1}, by intraperitoneal injection (i.p.)). Anaesthesia was maintained by the administration of further pentobarbitone as required. Cannulations were made with clear vinyl tubing (0.5 mm i.d.; 0.8 mm o.d., obtained from Dural Plastics, Dural, Australia). Urine was taken from the bladder at the end of acute experiments.

For enterohepatic recycling experiments, bile samples were collected up to 2 hr after intravenous (i.v.) [^{14}C]-BP administration. These bile samples containing radioactive BP metabolites (approximately 0.1 μmoles per ml) were infused (1 ml over a period of 1 hr) into the duodena of another group of anaesthetised rats with bile duct cannulae. For this procedure a second cannula was inserted, at the time of bile fistula surgery, into the duodenum via the bile duct. Rats were allowed to recover from the anaesthetic and were then restrained without food, but with water *ad libitum*, for a total period of 30 hr with provisions for the collection of urine and bile.

For the intermittent collection of bile from rats not under anaesthesia, a re-entrant cannulation technique [38] was used. Rats were allowed a post-operative recovery period of 10 days prior to injection of [^{14}C]-BP. Sodium pentobarbitone (50 mg kg^{-1}, i.p.) was administered to allow this injection to be carried out. Bile collections of 30 minute intervals were made at specified time points after [^{14}C]-BP injection.

[^{14}C]-BP was administered to rats by injection into the femoral vein (3 μmoles kg^{-1}; approx. 25 μCi kg^{-1}). In certain experiments, where biliary metabolites were being collected for use in enterohepatic recycling experiments, and in the case of rats with re-entrant bile duct cannulae, the specific radioactivity of [^{14}C]-BP was increased such that animals received 65 μCi kg^{-1}. [^{14}C]-BP was solubilised in Mulgofen® EL-719 (GAF Ltd., Tilson Road, Roundthorn, Wythenshawe, Manchester): ethanol (1:1 by volume). Distilled water was then added such that the final concentration of Mulgofen was 10%. Dose solutions of [^{14}C]-BP were at a concentration of 3 μmoles ml^{-1}.

Analysis of metabolites. Bile and urine samples (0.02 ml) or organic extracts and elutions from HPLC (up to 0.3 ml) were added to scintillation fluid (4 ml) for measurement of radioactivity. The scintillant consisted of 2,5-diphenyloxazole (5% w/v) and 1,4-di-2-(5-phenyloxazolyl)-benzene (0.3% w/v) in toluene (scintillation grade: BDH) mixed with Triton X-100 at a ratio of 2:1. Efficiency of counting was assessed using an external standard and reference to a quench-correction curve, constructed using [^{14}C]toluene (The Radiochemical Centre).

Samples of bile (0.2–1.0 ml) containing [^{14}C]-BP metabolites were either directly extracted with ethyl acetate (3 × 2 ml) or first incubated with β-glucuronidase (10,000 units per ml bile) with or without the addition of arylsulphatase (approx. 500 units per ml.). Control incubations contained 0.1 M sodium acetate buffer (pH 5) in place of the Ketodase. Incubations were carried out overnight in stoppered tubes held in a covered shaking water bath at 37°. Incubates were then adjusted to pH 7 with 0.2 M NaOH and extracted with ethyl acetate (3 × 5 ml). The ethyl acetate was evaporated to dryness with nitrogen and redissolved in 3 × 5 ml hexane: ethanol (4:1 by volume), which was passed through a silica column (SEP-PAK: Waters Associates, Hartford, Cheshire) previously washed with hexane (5 ml). The column was then eluted with hexane:ethanol (5 ml). The residue of the acetate extract was then added to the column in 200 μl methanol and eluted with a further 10 ml of hexane:ethanol. All hexane:ethanol eluates were pooled to give a total volume of 30 ml. The column was finally eluted with 10 ml of methanol. These two fractions accounted for approximately 95% of the ^{14}C in the ethyl acetate extracts. This procedure allowed considerable sample clarification and partial separation of BP metabolites in extracts of bile. The fractions were then evaporated to dryness with nitrogen, taken up in methanol (3 × 200 μl), and filtered using a sample clarification kit (Waters Associates).

Evaporation of solvents was carried out in subdued light and samples were stored at −20° when necessary.

High performance liquid chromatography (HPLC) analyses. The separation of BP metabolites by reversed phase HPLC has been previously described [39, 40] and similar conditions were employed here. Metabolites in ethyl acetate extracts were analysed using a Waters Associates HPLC equipped with Model 6000A solvent delivery systems, Model 660 solvent programmer and Model 440 U.V. absorbance detector (254 nm), equipped with a μ bondapak C_{18} column (3.9 mm i.d. × 30 cm). Samples were injected (U6K injector; Waters Associates) in methanol (50 μl), and eluted from the column with a linear gradient composed of 60–85% methanol in water, over a period of 40 min. The conditions were held at the end of the gradient for a further 15 min. The flow rate was 0.6 ml min^{-1}. Fractions of eluent were collected at 30 sec intervals into scintillation vial inserts (Hughes and Hughes Ltd., Romford,

Essex) using a 2112 Redirac fraction collector (LKB, S. Croydon, Surrey). The radioactive metabolites were identified by co-chromatography with authentic metabolites of BP and this identification can, therefore, be only tentative since related metabolites may, in certain instances, co-chromatograph in the HPLC systems currently available. 1-OHBP and 7-OHBP for example, have been detected as minor metabolites of BP when incubated with rat liver microsomes and these metabolites share very similar chromatographic characteristics to that of 3-OHBP [41].

RESULTS

Excretion of [^{14}C]-*BP metabolites in bile.* Following i.v. administration of [^{14}C]-BP to anaesthetised bile duct cannulated rats 58.6 ± 11.7 per cent ($n = 9$)* of the radioactivity was excreted in 6 hr via the bile with less than 3% in the urine. The metabolites in 0–2 hr bile samples were mainly in the form of polar conjugates since only 7.7 ± 0.4 per cent ($n = 2$) of the ^{14}C was directly extracted by ethyl acetate. After incubation of bile samples with β-glucuronidase, 38.4 ± 6.0 per cent ($n = 7$) of the ^{14}C was extracted as compared with 14.1 ± 0.1 per cent ($n = 2$) after control incubations. Following this treatment approximately 60 per cent of the biliary ^{14}C remained in the aqueous phase probably in the form of glutathione and other thioether conjugates. This fraction is under investigation.

Reversed phase HPLC analysis of the ethyl acetate extracts of bile treated with β-glucuronidase revealed several peaks of radioactivity in the column eluent which coincided in elution volume with authentic standards of BP metabolites (Fig. 1). The proportions of ethyl acetate-extractable ^{14}C co-chromatographing with these standards is shown in Table 1. Of the diols detected, BP 4,5-diol (peak D) was the most significant (16.8% of the ^{14}C) whereas BP 9,10-diol (peak B) represented less than 3 per cent of the activity and BP 7, 8-diol (peak D1) was not detectable above background levels. Radioactivity was also found to co-chromatograph with the phenolic benzo(*a*)pyrene metabolites: 3-OHBP (5.3%; peak H) and 9-OH BP (5.4%; peak G). A significant amount of BP 3,6-quinone (5.9%; peak F) was present together with trace amounts of BP 1,6-quinone, BP 6,12-quinone and BP itself. Two further small peaks (C and E) of unidentified radioactivity were found together with a major fraction (peak A) eluting with the solvent front and comprising 38 per cent of the extractable ^{14}C. The latter probably represents a mixture of conjugates and/or polyhydroxylated metabolites such as triols or tetrols, which are fairly polar in nature since the major proportion of this fraction was eluted from the 'SEP-PAKS' only with methanol (see Materials and Methods). The metabolite profiles found after incubating bile fractions with a mixture of aryl sulphatase and β-glucuronidase were qualitatively and quantitatively similar to those found with β-glucuronidase alone, thus giving no indication of the presence of significant quantities of sulphate conjugates.

Enterohepatic circulation of biliary metabolites. Rat bile, collected up to 2 hr after administration of [^{14}C]-BP (i.v.), was infused into the duodena of a second group of bile duct cannulated rats when 18.7 ± 6.5 per cent ($n = 7$) of the dose was re-excreted via the bile within 30 hr (Fig. 2). A small

* Data are mean ±S.D. Figures in parentheses are the number of animals used.

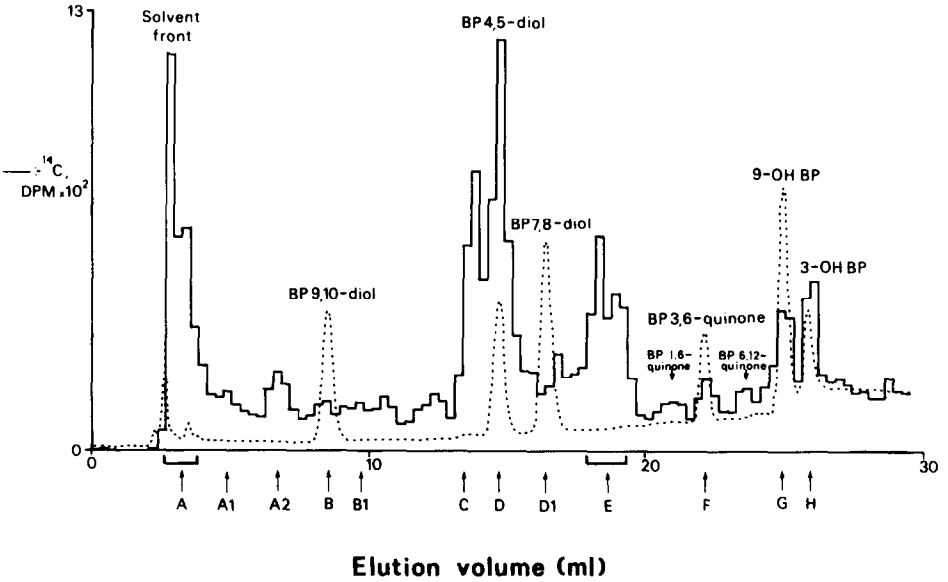

Fig. 1. Reversed phase high performance liquid chromatographic analysis of BP metabolites. The specific conditions for this separation are described in the text. Dashed trace shows U.V.$_{254}$ absorption of standards as indicated. A to H indicate the elution pattern of the biliary metabolites of [^{14}C]-BP in the rat (see text). The solid line shows a representative profile of radioactivity extracted with ethyl acetate from 2 hr rat bile and eluted from a silica column with hexane : ethanol (4 : 1); see Methods.

Table 1. Reversed phase high performance liquid chromatography (HPLC) of ^{14}C-labelled benzo(*a*)pyrene (BP) metabolites excreted as glucuronic acid conjugates in rat bile

Radioactive region eluted from HPLC column*	Nature of metabolite*	Per cent of ^{14}C in ethyl acetate extract† of bile (mean ± S.D.; n = 5)
A	Conjugates and/or polyhydroxylated metabolites	37.5 ± 4.8
B	BP 9,10-diol	<3.0
C	unknown	5.6 ± 4.6
D	BP 4,5-diol	16.8 ± 3.5
D1	BP 7,8-diol	not detected (<1.0)
E	unknown	7.2 ± 3.1
F	BP 3,6-quinone	5.9 ± 8.1
G	9-OHBP	5.4 ± 1.7
H	3-OHBP	5.3 ± 0.5

* See Fig. 1.

† [^{14}C]-BP was administered i.v. (3 μmoles kg^{-1}) to bile duct cannulated rats and bile collected for 2 hr. Metabolites were extracted into ethyl acetate following incubation of bile with β-glucuronidase (see text). Traces of ^{14}C eluting with BP 1,6-quinone, BP 6,12-quinone and BP itself were also found together with several other minor metabolites, which individually did not account for more than 3% of the ^{14}C in the extract, and which did not co-chromatograph with any of the authentic standards available.

proportion of the dose (1.3 ± 0.7%; n = 7) was excreted in the urine. The rate of excretion of recycled metabolites in rat bile appeared to fall in each animal at around 10 hr after the start of infusion but rose again consistently at around 24 hr.

Bile samples were taken from two sections of the re-excretion profile ((a) 0–10 hr and (b) 20–30 hr) and incubated with β-glucuronidase. After this treatment, 41.2 ± 7.9 and 36.7 ± 3.5 per cent (n = 3) respectively of the ^{14}C in these two fractions of bile was extractable into ethyl acetate at pH 7. The extracts were analysed as described earlier and the results are shown in Table 2. A very similar pattern of metabolites to that in bile collected after the i.v. administration of [^{14}C]-BP (Table 1) was found. Individual variation, between animals, in the proportion of some metabolites (particularly 3-OHBP and BP 3,6-quinone) is reflected in the large S.D. values. This may, in part, be a result of a variable extent of oxidation during sample preparation for analysis, since 3-OHBP is readily oxidised to BP 3,6-quinone [42]. However, the pattern of metabolites appeared the same when the β-glucuronidase hydrolyses were carried out under nitrogen to minimise oxidation. It is noteworthy that no BP 7,8-diol was detected in the bile in these enterohepatic circulation experiments.

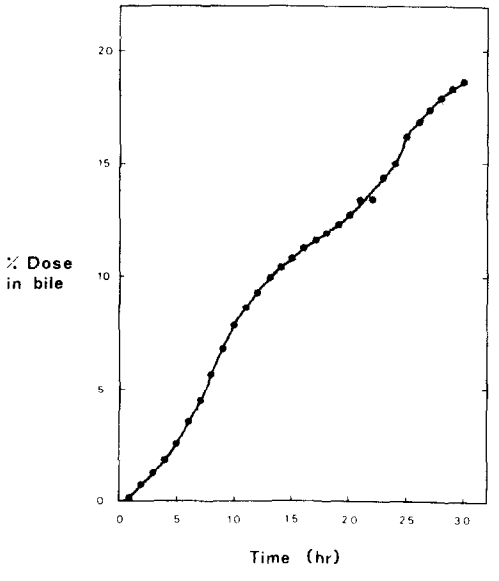

Fig. 2. Enterohepatic circulation of [^{14}C]-BP metabolites in the rat. Biliary metabolites of [^{14}C]-BP were infused intraduodenally (see text) over a period of 1 hr into the bile duct-cannulated rats, and the excretion of ^{14}C in the bile monitored. The shaded area represents S.D. (n = 7).

BP metabolites in chronic intermittent collections of bile. In order to examine the persistence of BP metabolites in rat bile, with minimal interference to the enterohepatic circulation, the level and nature of ^{14}C was investigated, over a period of seven days, in bile samples taken intermittently after a single i.v. dose of [^{14}C]-BP was administered to rats with re-entrant bile duct cannulae. The total level of BP metabolites in the bile of two rats is shown in Fig. 3, and the percentage of this total ^{14}C extracted into ethyl acetate, following incubation with β-glucuronidase, ranged from 30 to 48 per cent at different time points (Table 3). The level of ^{14}C in bile taken more than two days after injection of BP was not sufficient to allow quantification of individual metabolites, and after day 7, it fell below the limit of detection. As time after BP administration increases, a change is seen in the relative proportion of certain metabolites. The metabolite fraction G, tentatively identified as 9-OHBP, was not detected in bile samples taken after one hour, yet the relative size of peak H, which co-chromatographed with 3-OHBP, was elevated at

Table 2. Reversed phase high performance liquid chromatography (HPLC) of enterohepatic-recycled biliary metabolites of ^{14}C-labelled benzo(*a*)pyrene (BP) in the rat

Radioactive region eluted from HPLC column*	Nature of metabolite*	Per cent of ^{14}C in ethyl acetate extract† (mean ± S.D.; n = 3) of: (a) 0–10 hr bile	(b) 20–30 hr bile
A	Conjugates and/or polyhydroxylated metabolites	26.8 ± 3.8	36.0 ± 5.1
A1	unknown	5.7 ± 0.9	6.0 ± 1.1
B	BP 9,10-diol	<3.0	<3.0
C	unknown	9.3 ± 4.5	9.0 ± 2.7
D	BP 4,5-diol	12.8 ± 3..3	19.7 ± 3.1
D1	BP 7,8-diol	not detected (<1.0)	not detected (<1.0)
E	unknown	3.9 ± 1.8	14.2 ± 7.5
F	BP 3,6-quinone	5.7 ± 8.5	6.1 ± 6.3
G	9-OHBP	2.2 ± 1.4	4.6 ± 4.6
H	3-OHBP	4.7 ± 4.2	2.9 ± 2.4

* See Fig. 1.

†Bile containing metabolites of [^{14}C]-BP was infused intraduodenally into bile duct cannulated rats and their bile collected for 30 hr to assess the extent of enterohepatic recycling of BP and its metabolites (see Fig. 2). Bile was analysed at (a) 0–10 hr and (b) 20–30 hr for BP metabolites, which were extracted into ethyl acetate following incubation of bile with β-glucuronidase (see text).

the 6.5 and 22 hr points. metabolite D1 (BP 7,8-diol) was detected at 1, 6.5, 22 and 32 hr and appeared to be increased in relative importance to other metabolites at 6.5 hr.

DISCUSSION

The bile of the rat is a major route of excretion for BP metabolites in accordance with previous reports [29, 30]. Only a small percentage (8%) of total radioactivity in bile could be directly extracted into ethyl acetate indicating that the majority of metabolites are polar conjugates. This ethyl acetate-soluble fraction may have included trace amounts of sulphate conjugates e.g. BP-3yl-sulphate, which is known to be formed from BP by rat hepatocytes [26]. However, the pattern of metabolites extracted into ethyl acetate did not appear to alter when bile samples were hydrolysed with aryl sulphatase together with β-glucuronidase as compared with β-glucuronidase alone. It may be that BP-3yl-sulphate, if formed *in vivo*, is not readily excreted due to its relatively high lipophilicity [17].

A large proportion of the metabolites was extractable into ethyl acetate after β-glucuronidase hydrolysis. This again agrees with previous findings where

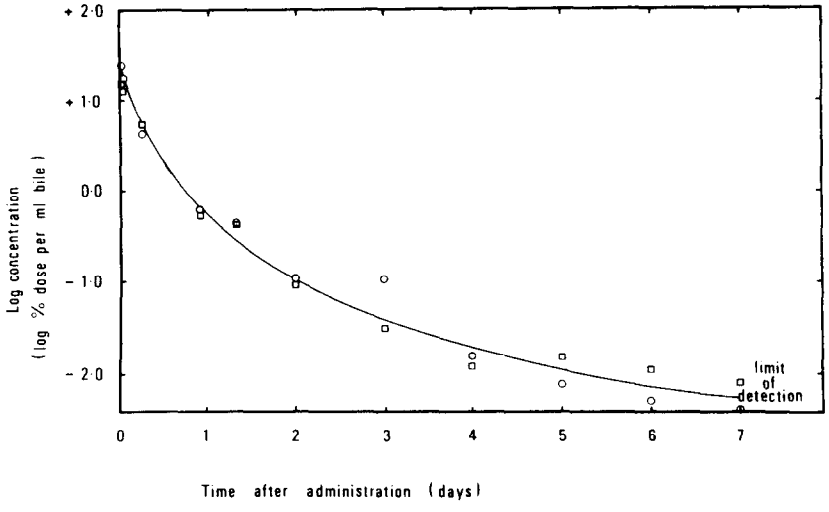

Fig. 3. Persistence of [^{14}C]-BP metabolites in rat bile. Bile samples were taken intermittently over a period of 7 days from rats with re-entrant bile duct-cannulae (see text), following a single i.v. dose of [^{14}C]-BP (3 μmoles kg^{-1}). Results are expressed as log biliary ^{14}C concentration vs time for two rats (○ and □).

glucuronic acid conjugates of BP metabolites were excreted in rat bile [30, 43]. Metabolites which remain in the aqueous fraction following this treatment are possibly glutathione and other thioether conjugates. BP 4,5-oxide, for example, is excreted partially as thioether conjugates in the bile of rats and of perfused rat liver [43, 44]. It is also possible that certain glucuronic acid conjugates, which are resistant to hydrolysis by β-glucuronidase, may be present in this fraction. Such conjugates are possibly formed from BP in the perfused rabbit lung [45], although they have not yet been fully characterised.

Amongst the major metabolites found after enzymatic hydrolysis of glucuronic acid conjugates in rat bile were BP 4,5-diol and 3-OHBP. These metabolites are also major products formed by rat hepatocytes [26], are found in the fæces of rats given [^{14}C]-BP by gavage (46) and were identified as biliary metabolites of BP in the earlier study of Falk *et al.* [30]. BP 7,8-diol was not detected in β-glucuronidase hydrolysates of 2 hr rat bile samples, but in experiments where chronic intermittent bile collections were made, ^{14}C which co-chromatographed with BP 7,8-diol was detected in significant amounts. Hecht *et al.* [46] have also detected trace amounts of this diol as a faecal metabolite in the rat. Isolated hepatocytes form the 4,5-, 7,8- and 9,10- diols from BP [26] but the latter two diols are found in the form of glucuronides in only trace amounts, this may be related to the low rate of glucuronic acid conjugation of these diols compared with that of the 4,5-diol [47]. This could explain the relative predominance in the glucuronide fraction of rat bile of the 4,5-diol compared with the 7,8- and 9,10-diols observed in our experiments. Of particular interest is the question of whether the proximate carcinogen BP 7,8-diol is capable of reaching the systemic circulation as a consequence of the apparent inefficiency of its conjugation in the liver. It is interesting to note that in the perfused rat liver, a significant amount of unconjugated BP 4,5-diol, formed from BP 4,5-oxide, was released into the perfusion medium [44]. BP 7,8-diol can also be converted to 7,8,9,10-tetrahydro 7,8,9,10-tetrahydroxy BP as has been shown to occur with rat hepatic microsomes [48] and it is possible that this polar metabolite is present in bile, though not identified, in fractions A, A1, or A2.

In contrast to the rat the glucuronic acid conjugate of BP 9,10-diol has been identified as a major biliary metabolite of BP in the rabbit [31] and unpublished results). The basis for this marked species difference is unknown but may be a reflection of a difference in the regio-specificity of oxidation and/or in a difference in the ability to glucuronidate and excrete BP 9,10-diol.

Biliary metabolites of BP were found to undergo enterohepatic circulation. The pattern of metabolites in the bile of rats which had received an intraduodenal infusion of biliary metabolites was very similar to that in the infused bile. An additional metabolite (Fraction A1) was however found in recycled bile extracts and also in bile collected from the animals with re-entrant bile duct cannulae where it appeared to increase in relative amount with time (see Table 3). This could be a relatively polar metabolite such as a triol or tetrol derived by further metabolism of a primary metabolite and the appearance of "secondary" metabolites in the bile might be expected

Table 3. Reversed phase high performance liquid chromatography (HPLC) of ^{14}C-labelled benzo(*a*)pyrene (BP) metabolites present in chronic intermittent collections of rat bile

Radioactive region eluted from HPLC column*	Nature of metabolite	Per cent of ^{14}C in ethyl acetate extract† of bile collected at: 1 hr rat 1, rat 2	6.5 hr rat 1, rat 2	22 hr rat 1 + 2	32 hr rat 1 +2	48 hr rat 1 +2	72 hr rat 1 +2
A	Conjugates and/or polyhydroxylated metabolites	33.1,39.6	28.0,22.0	29.5	32.6	–	–
A1	unknown	2.3, 0.3	2.8, 3.3	7.3	7.2		
A2	unknown	2.3, 1.2	1.1, 1.6	1.1	0.7	–	–
B1	unknown	1.2, 2.9	3.5, 2.2	5.0	2.0	–	–
C	unknown	8.5, 7.7	9.2, 7.9	5.0	6.7	+	+
D	BP 4,5-diol	18.4,12.8	12.1, 9.5	11.9	9.3	+	+
D1	BP 7,8-diol	1.5, 3.0	5.8, 7.7	approx. 4	approx. 2	–	
E	unknown	6.9, 8.8	9.6, 9.8	approx. 17	approx. 10	+	+
F	BP 3,6-quinone	0.8, 0.8	3.0, 2.6	1.0	5.5	–	–
G	9-OHBP	3.2, 3.8	<1, <1	<1	<1	–	–
H	3-OHBP	5.2, 5.4	15.9, 8.9	17.5	4.0	–	–
% of ^{14}C in bile extractable into ethyl acetate		35, 37	44, 48	31	30		

* See Fig. 1.

† A single i.v. dose of [^{14}C]-BP (3 μmoles kg^{-1}) was administered to rats with re-entrant bile duct cannulae (see text) and bile collected for 30 min at each of the time points shown. Metabolites were extracted into ethyl acetate following incubation of bile with β-glucuronidase (see text). Results are for two rats; No. 1 (○ in Fig. 3) and No. 2 (□ in Fig. 3). The level of ^{14}C in bile at 48 and 72 hr was too low for quantification of metabolites; + means metabolite present, − means metabolite not detected.

particularly at later time points. In animals with re-entrant cannulae there was also an increase in the relative amounts of conjugated 3-OHBP in rat bile at the 6.5 hr and 22 hr time points. A delay in the biliary excretion of this metabolite was also noted by Falk *et al.* [30] during a study over a shorter time period with anaesthetised rats. In contrast, the excretion of 9-OHBP (peak G; Table 3) was only observed at the 1 hr time point.

This study has demonstrated that metabolites of BP are excreted via the bile of the rat and are capable of being reabsorbed from the intestine to undergo enterohepatic circulation. The systemic availability of free metabolites from the intestine will depend upon the extent of extraction by the liver. Since a small portion of the metabolites administered to the duodenum is excreted via the urine, it follows that some of the recycled metabolites do reach the systemic circulation. Human colonic tissue was found to mainly utilise sulphate to conjugate BP metabolites [48]. However, sulphate conjugates of BP could not be detected in the bile of rats which had received an intraduodenal infusion of BP metabolites. It is possible that, if such conjugates were formed in the intestinal wall, they may have been retained by the body. Thus metabolites which are released directly from the liver or which escape from the enterohepatic circulation, could become available to other body tissues which may be susceptible to BP-induced carcinogenesis.

Autrup *et al.* [36] have shown that the cultured human colon can metabolise BP to BP 7,8-diol and that this diol can be subsequently converted to the ultimate carcinogen, BP 7,8-diol, 9,10-epoxide which binds covalently to the DNA of this tissue. The process of enterohepatic circulation necessitates the transfer of biliary metabolites through the intestinal wall. Since conjugates of BP 7,8-diol appear to be present in rat bile, along with other potentially toxic metabolites, it is possible that these products could be further metabolised and ultimately react with nucleophilic constituents of intestinal cells. In this regard the observation that mutagenic metabolites of BP are excreted in rat bile [33] may be of particular significance.

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