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THE METABOLISM OF FOREIGN COMPOUNDS IN RATS AFTER TREATMENT WITH POLYCHLORINATED BIPHENYLS (PCBs)*

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Abstract—Pretreatment of rats with a single dose of polychlorinated biphenyls caused an increase in the activity of UDP glucuronyltransferase (EC 2.4.1.17; acceptor unspecific) in liver microsomes towards the substrates *p*-nitrophenol and 4-methylumbelliferone. The results of kinetic experiments suggest enhanced enzyme activity is caused by enzyme induction. The effect was dependent on dose and chlorine content of the polychlorinated biphenyls administrated. The time course of UDP glucuronyltransferase activity after a single dose of polychlorinated biphenyls demonstrated a long lasting effect. Elevated biliary excretion of sulfobromophthalein conjugates after pretreatment with polychlorinated biphenyls was also demonstrated. In addition bile flow per g liver was increased and sulfobromophthalein concentration in serum and liver was reduced. Maximum effects were observed three days after treatment; thereafter these parameters slowly returned to normal levels.

Many lipophilic compounds must be metabolically converted to polar compounds for excretion. In most cases these reactions are mediated by enzyme systems in hepatic endoplasmic reticulum: hydroxylation by monooxygenases, and subsequently often conjugation to glucoronic acid by UDP glucuronyltransferase (EC 2.4.1.17). The resulting metabolites are excreted into bile or urine depending on their molecular weight [1]. As described previously, polychlorinated biphenyls (PCBs) like phenobarbital and organochlorine insecticides, are potent inducers of microsomal monooxygenases [2, 3].

The main purpose of this study was to investigate whether second step reactions of drug metabolism in the liver, glucuronidation and glutathione conjugation, were also affected by PCBs. An increased rate of glucuronidation could be responsible for the reported rise of excretion of polar steroid metabolites *in vivo* [4, 5].

The activity of microsomal UDP-glucuronyltransferase was measured using *p*-nitrophenol and 4-methylumbelliferone as substrates. Biliary excretion of BSP and its conjugates was measured to elucidate whether PCBs increase conjugation reactions which occur in the soluble fraction of liver cells.

MATERIALS AND METHODS

PCBs were a generous gift from Monsanto (Germany). The products used in the experiments were mixtures of isomers containing predominantly dichlorobiphenyls (Aroclor® 1232 = di-CB), tetrachlorobiphenyls (Aroclor® 1248 = tetra-CB) and hexachlorobiphenyls (Aroclor® 1260 = hexa-CB).

UDP-glucuronate was purchased from Boehringer (Mannheim), 4-methylumbelliferone from Serva (Heidelberg). All other chemicals were of analytical grade and obtained from Merck (Darmstadt).

Male Wistar rats weighing 200 ± 10 g were given a single intraperitoneal injection of PCBs (dissolved in arachis oil, 1 ml/kg; see text for dose details) between 8 a.m. and 9 a.m. Control rats received an equal volume of arachis oil i.p. Animals were fed *ad libitum* on a normal laboratory diet (Altromin®/Lage/Lippe, Germany).

Preparation of microsomes. Rats were sacrificed by decapitation between 8 a.m. and 9 a.m. at the intervals after treatment indicated in the table and figure legends. Livers were perfused through *a. hepatica* (via aorta) with ice-cold NaCl (0·9% w/v), excised and cooled on ice. The microsomal fraction was prepared from 8 g liver as described previously [8], and stored in small aliquots at −20°. No loss of activity was detectable after storage for 7 weeks at −20°. Protein was determined by the method of Lowry *et al.* [9], using bovine serum albumin as a standard.

Enzyme assays. The glucuronidation of *p*-nitrophenol was assayed in an incubation system containing in a total volume of 0·25 ml: 50 mM Tris–HCl pH 7·5, 150 mM KCl, 0·05% Triton X-100, 10 mM $MgCl_2$, 0·6 mM *p*-nitrophenol, 0·1–0·2 mg microsomal protein. After preincubation for 5 min at 37° the reaction was started by the addition of UDP glucuronate (final concentration 3·2 mM). Aliquots of 0·1 ml were removed from the assay tubes at different times (3–5 min), and added to 2·0 ml of 2% trichloroacetic acid (w/v) in order to stop the reaction. After centrifugation for 10 min at 3000 *g* to remove precipitated protein, the supernatant was adjusted to pH > 10 with 5N KOH, and the optical density was measured at 403 nm. Blanks were obtained from incubations without UDP glucuronate or without microsomes.

* Some of these results were reported at the Spring Meeting of the Deutsche Pharmakologische Gesellschaft, Mainz 1972 [26].

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Abbreviations: BSP, sulfobromophthalein sodium; di-CB, dichlorobiphenyls; GSH, glutathione; *hexa-CB*, hexachlorobiphenyl; PCBs, polychlorinated biphenyls; tetra-CB, tetrachlorobiphenyls.

The glucuronidation of 4-methylumbelliferone in microsomes activated by Triton X-100 (0·05%, 5 min at 37°) was measured spectrofluorometrically by the method of Frei *et al.* [10] (Zeiss, excitation 369·5 nm, emission 449·5 nm).

Sulfobromophthalein excretion. The rats were pretreated with a single dose of 1·7 m-moles tetra-CB/kg (dissolved in arachis oil). After various time periods the animals were anaesthetized with urethane. The *a. carotis*, *v. jugularis*, and the bile duct were cannulated with polyethylene tubing. The rectal temperature was maintained at 37° by a heat lamp. Before administration of BSP three 10-min bile samples were collected. After this a BSP infusion, sufficient for BSP-T_m-measurements (3·4 m-moles/kg × min), was administered over a 60-min period into the *v. jugularis*. Bile samples were collected at 10-min intervals. Blood samples were obtained from the cannulated *a. carotis*.

The volume of bile samples was determined gravimetrically (assumed density = 1·0 g/ml). Concentrations of total BSP were measured spectrophotometrically (at 575 nm) in plasma or bile after dilution with appropriate amounts of 0·1 N NaOH. BSP-T_m [maximal biliary excretion (μmoles/kg × min)] was calculated from the 10-min bile fraction with the highest content of total BSP. Unconjugated and conjugated BSP were determined by the thin-layer chromatography method of Whelan and Plaa [11]. The total BSP content of the liver after 60 min of BSP infusion was determined by the method of Whelan *et al.* [12].

Phenolphthalein excretion. 4 days after treatment with tetra-CB (1·7 m-moles/kg) rats were anaesthetized with urethane, and phenolphthalein was administered by a rapid intravenous injection (0·157 m-moles/kg, as the sodium salt dissolved in 2·5 ml NaCl 0·9%. Thereafter two 30-min bile samples were collected. The content of phenolphthalein and phenolphthalein glucuronide was determined spectrophotometrically (at 550 nm) by the method of Millburn *et al.* [1].

The statistical significance was determined by Student's *t*-test. The levels of significance are given in the table and figure legends.

RESULTS

Activity of UDP-glucuronyltransferase. In order to study the effects of pretreatment of PCBs on glucuronide conjugation *in vivo* the biliary excretion of phenolphthalein was measured. This compound is excreted almost completely as its glucuronide. In rats no significant increase in biliary excretion of total phenolphthalein (dose 0·157 m-moles/kg i.v.) was found after administration of tetra-CB (controls 24·8%, S.D. = ±3·5; tetra-CB treated 28·2%, S.D. = ±6·5 of the administered dose per 30 min; n = 8). *In vivo* the rate limiting step in biliary excretion of phenolphthalein is the transport process of the formed glucuronide into the lumen of the bile canaliculus rather than the activity of UDP glucuronyltransferase [13]. To investigate glucuronide conjugation, apart from transport phenomena, of the intact liver cell the activity of the UDP-glucuronyltransferase was measured *in vitro* after treatment of rats with PCBs. Because the activity of UDP-glucuronyltransferase in microsomes is increased by treatments affecting the membrane structure ('ageing' [14], detergents [14], phospholipase-A [15]) the enzyme assays were performed with Triton X-100 0·05% to obtain maximum activity [14].

Table 1. Effect of tetra-CB* treatment on kinetic parameters of UDP-glucuronyltransferase

	Controls	Tetra-CB treated*
K_m UDP glucuronic acid (mM)	1·61	1·61
K_m *p*-nitrophenol (mM)	0·29	0·29
V_{max} *p*-nitrophenol†	140	580

Apparent K_m- and V_{max}-values were calculated from Lineweaver–Burk plots using the regression line of mean values of 4–6 animals. Substrate concentrations varied from 0·4 to 3·2 mM for UDP glucuronic acid, and from 0·125 to 1·0 mM for *p*-nitrophenol.

* 1·7 m-moles tetra-CB/kg i.p.; sacrificed 4 days later.

† nmoles pNP-glucuronide formed/mg microsomal prot. × min.

The glucuronide production in microsomes of controls and treated animals was linear for up to 3–5 min under assay conditions used. The values given in the tables and figures were calculated from 3-min incubations.

As shown in Table 1, pretreatment of rats with PCBs does not affect the K_m of the conjugation reaction with glucuronic acid whereas the V_{max} is four times higher in treated animals than in control animals. This was interpreted as induction of UDP-

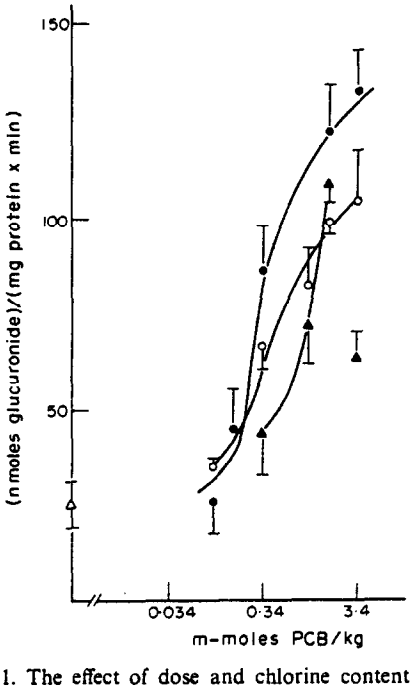

Fig. 1. The effect of dose and chlorine content of PCBs on activity of UDP-glucuronyltransferase using *p*-nitrophenol as substrate. Experiments were performed 3 days after administration of di-CB (▲), and 4 days after administration of tetra-CB (●) and hexa-CB (○). For experimental details see Materials and Methods. The values are means ±S.D. of results from 4 to 6 animals.

Table 2. Effect of dose and chlorine content of PCBs on the activity of UDP-glucuronyltransferase using 4-methylumbelliferone as substrate

Tetra-chlorobiphenyl (m-moles/kg)	Hexa-chlorobiphenyl (m-moles/kg)	4-methylumbelliferone-glucuronide (nmoles/mg microsomal prot. × min)
—	—	28·3 ± 6·0
0·33	—	61·1 ± 7·7†
1·0	—	80·0 ± 7·3*
3·33	—	116·6 ± 5·7†
—	0·33	72·2 ± 5·8†
—	1·0	94·7 ± 7·0†
—	3·33	98·6 ± 0·7 N.S.

Enzyme activities were determined 4 days after treatment of rats with a single dose of PCBs.

For experimental see Materials and Methods.

Values are means ± S.D. of 4–6 rats. Significant differences from the lower dose are designated as: † = $P < 0.01$, * = $P < 0.05$. N.S. = difference not significant.

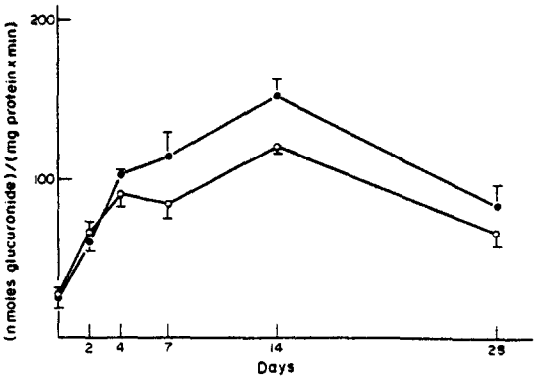

Fig. 2. UDP-glucuronyltransferase activity at different intervals after a single injection of 1·7 m-moles tetra-CB/kg. (●) substrate *p*-nitrophenol, (○) substrate 4-methylumbelliferone. Enzyme activities were determined as mentioned under Materials and Methods. The values are means ± S.D. of results from 4 to 6 animals.

glucuronyltransferase and led to an investigation of the dependence of the increased enzyme activity on dose and chlorine content of the administrated PCBs.

The enzyme activity was measured at a time after administration of PCBs when maximum activity of the mono-oxygenases was observed in liver microsomes (2–4 days with respect to dose and chlorine content [8]). UDP glucuronyltransferase activity, however, was maximal at 14 days, see below.

As indicated in Fig. 1 the threshold dose of tetra-CB is 0·17 m-moles/kg and the maximum effect (134 nmoles *p*-nitrophenol glucuronide/mg microsomal protein × min) is obtained at 3·4 m-moles/kg. This is a rise of enzyme activity of 4·5 times that of controls. Nearly the same rate of glucuronide formation was obtained at a dose of 1·7 m-moles/kg. The induction of UDP-glucuronyltransferase by di-CB and hexa-CB was different. Hexa-CB was found to have a lower threshold dose (0·1 m-mole/kg) but the maximum effect was not as high as that of tetra-CB. The dose response curve of di-CB is shifted to higher dosages and is of similar steepness as that of tetra CB. However, at doses higher than 1·7 m-moles/kg an abrupt decrease of activity was found.

With 4-methylumbelliferone as substrate the increase of activity was not as high as it was with *p*-nitrophenol (Table 2) but showed a similar pattern with respect to dose and chlorine content of the PCBs.

The most effective induction was obtained by tetra-CB. Therefore the time course of action of this PCB mixture was studied. The activity of the UDP-glucuronyltransferase was measured at various times up to 4 weeks after a single dose of 1·7 m-moles/kg (Fig. 2). Unexpectedly maximum activity was seen after 14 days, instead of 4 days found in studies of the induction of mixed function oxidases [3]. Even 4 weeks after treatment the activity of UDP-glucuronyltransferase was 3·5 times that of controls. A less marked but otherwise similar course of activity with respect to time was obtained using 4-methylumbelliferone as substrate.

Biliary BSP excretion. The elimination rate of BSP into bile depends mainly on the rate of conjugation with GSH by *S*-aryltransferase [12, 16, 17]. High liver concentrations of BSP can be obtained by i.v. infusion leading to, presumably, saturating substrate concentrations for the S-aryltransferase *in vivo* [17]. In order to investigate whether the activity of this conjugation reaction was increased after treatment of rats with PCBs the biliary excretion of free BSP and its conjugates was measured following constant BSP infusion of 3·4 m-moles/kg × min.

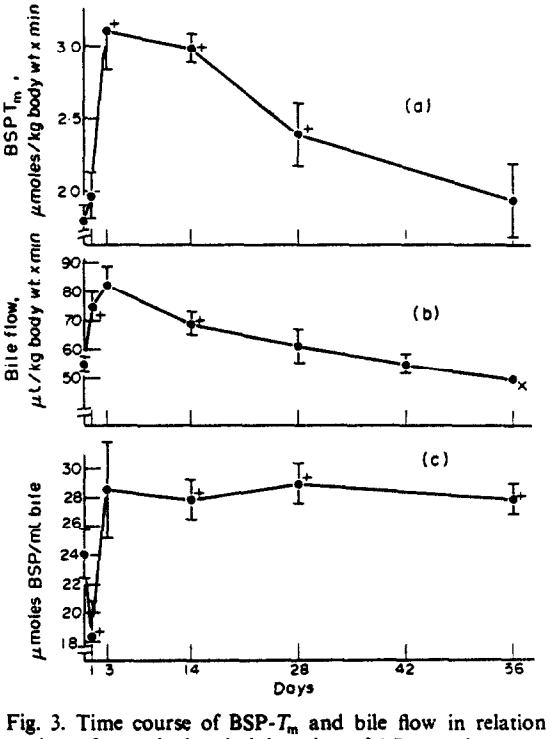

Fig. 3. Time course of BSP-T_m and bile flow in relation to time after a single administration of 1·7 m-moles tetra-CB/kg. The values are means ± S.D. of 4 to 6 rats. (a) T_m values (mg total BSP/kg × min), (b) bile flow before start of BSP infusion, (c) concentration of total BSP in bile at the time of maximal biliary excretion. For experimental details see Materials and Methods. + = significant difference $P < 0.05$.

Figure 3 shows the values of maximal elimination rates of total BSP (BSP-T_m) at various times up to 8 weeks after administration of a single dose of 1·7 m-moles tetra-CB/kg. Maximal T_m values were observed on the third day after treatment (190 per cent of controls), then BSP-T_m decreased slowly. By 8 weeks T_m values had returned to normal levels. The increase of BSP-T_m during this period was caused by an increased biliary excretion of conjugated BSP (with GSH) alone, whereas the output of unconjugated BSP always remained within the normal range (0·27 ± 0·04 μmoles/kg × min). On the third day after treatment, when maximal T_m values were observed, we found a T_m of total BSP of 3·11 ± 0·28 μmoles/kg × min (normal range: 1·77 ± 0·16 μmoles/kg × min) consisting of 2·84 μmoles/kg × min BSP conjugates (normal range: 1·50 ± 0·12 μmoles/kg × min) and 0·27 ± 0·04 μmoles/kg × min unconjugated BSP (normal range: 0·26 ± 0·03 μmoles/kg × min). The bile flow (before BSP-infusion) was also increased and paralleled the time course of BSP-T_m. The elevation of both the BSP-T_m and the bile flow cannot be explained by liver growth alone because values expressed per g liver also show an increase (Fig. 4). Another indication of the increased conjugating activity may be the reduced BSP serum concentration [determined at the time of maximal biliary excretion of BSP (T_m)] and decreased BSP concentration in the liver (Fig. 4).

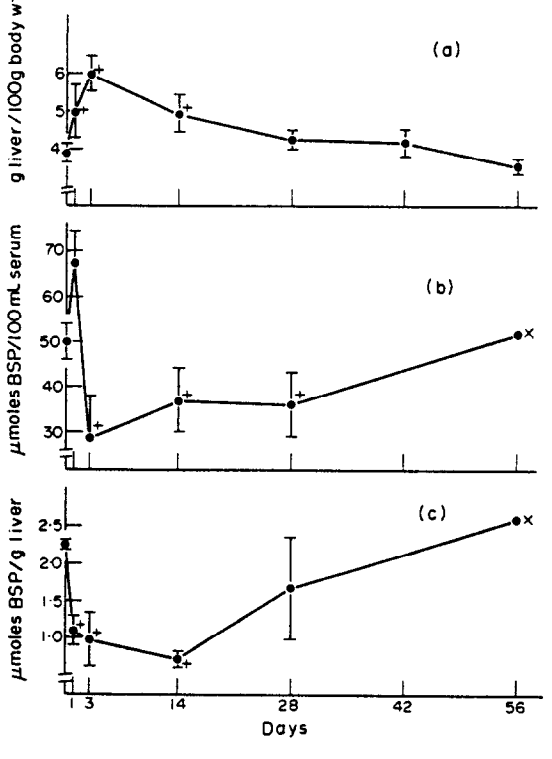

Fig. 4. Relative liver weight (g liver/100 g body wt.) (a), BSP concentration per 100 ml serum at the time of maximal biliary excretion (b), and BSP concentration per g liver (after 60 min of BSP infusion, 2·5 mg/kg × min) (c) in relation to time after a single dose of 1·7 m-moles tetra-CB/kg. Means ± S.D. of 4 to 6 rats. + = significant difference. × = means of 2 rats.

DISCUSSION

After treatment of rats with PCBs a dose dependent induction of microsomal mono-oxygenases and liver hypertrophy were observed [3, 8, 18, 19]. The long lasting effects of PCBs on enzyme activities are similar to those of organochlorine insecticides whereas enzyme induction by phenobarbital is more transitory. The long lasting effects are probably due to the high persistence of PCBs in the organism [20, 21] maintaining increased enzyme activities for a longer period (a "new steady state" [22]).

Our results show that another step of drug metabolism, the conjugation of GSH with glucuronic acid, is accelerated in a similar manner after treatment with PCBs. The induction of the microsomal UDP-glucuronyltransferase is dose dependent and yields higher activities after tetra-CB and hexa-CB treatment than after treatment with di-CB. Maximal enzyme activities following PCB administration are seen after 3 to 4 days for the mono-oxygenase system and after 2 weeks for UDP-glucuronyltransferase (Fig. 2). This indicates different mechanisms of induction as suggested earlier by Aitio [23]. Probably the induction of UDP-glucuronyltransferase parallels endoplasmic reticulum proliferation whereas the induction of mono-oxygenases precedes membrane proliferation (unpublished observations).

Conjugation of BSP with GSH is catalysed by the soluble enzyme *S*-aryltransferase which seems to be independent of membranes and on the process of membrane proliferation. The observation that only conjugated BSP was excreted into bile in increased amounts following treatment with PCBs also suggests an induction of this enzyme. This is in agreement with the results of Freundt [24], who found an increase of BSP conjugating activity *in vivo* and *in vitro* after pretreatment of rats with the inducing agent phenobarbital. Maximal activity 3 days after a single dose of PCBs corresponded to the induction of microsomal mono-oxygenases.

The increase in bile flow and the reduction of BSP concentration in serum and liver are similar to the effects produced by phenobarbital [6, 24]. However, the inverse levels of the BSP concentrations on the first day after PCB administration were not observed in experiments with phenobarbital and remain unexplained.

In spite of the similarities of induction by PCBs and phenobarbital one cannot conclude that all the metabolic pathways are influenced in the same manner by these two drugs, e.g. digitoxin metabolism *in vivo* is inhibited by PCBs but enhanced by phenobarbital [25].

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