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METABOLISM OF 4-CHLOROBIPHENYL BY GUINEA PIG ADRENOCORTICAL AND HEPATIC MICROSOMES

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Abstract—Studies were carried out to determine if 4-chlorobiphenyl (4-CB) was a substrate for adrenal monooxygenases and to compare its interactions with adrenal and hepatic microsomal enzymes. Addition of 4-CB to guinea pig adrenal microsomes produced a typical type I spectral change, indicative of binding to cytochrome(s) P-450 and similar to that seen in hepatic microsomal preparations. The activities of several adrenal and hepatic microsomal monooxygenases were decreased by 4-CB *in vitro*. High pressure liquid chromatographic analyses revealed that both adrenal and hepatic microsomes, in the presence of NADPH, converted 4-CB to a major metabolite which eluted with a retention time identical to that of 4-chloro-4′-biphenylol (4′-OH-4-CB). The identity of 4′-OH-4-CB was confirmed by mass spectrometry. The maximal rate of 4-CB metabolism was greater in adrenal, compared with liver microsomes, but 4-CB had a higher affinity for hepatic than for adrenal enzymes. The rate of adrenal 4-CB metabolism was four to five times greater in microsomes derived from the inner cortical zone (zona reticularis) than those from the outer zones (zona fasciculata and zona glomerulosa). Hepatic microsomes also converted 4-CB to a minor metabolite whose production was blocked by epoxide hydrolase inhibitors, suggesting it might be a diol. 4-CB metabolism was not demonstrable in adrenal mitochondrial preparations. The results indicate that chlorinated biphenyls can serve as substrates for adrenal microsomal monooxygenases, suggesting that local activation may contribute to their adrenocortical toxicity.

Polychlorinated biphenyls (PCBs) are widespread and persistent environmental contaminants that have been detected in various species of wildlife as well as in human tissues and milk [1–4]. As a result of their lipophilic nature, PCBs tend to accumulate in tissues having a high lipid content such as adipose tissue, liver, and adrenal glands [5–7]. The liver is the principal target organ for PCB-induced toxicities [8, 9]. PCBs cause numerous changes in hepatic function including induction of microsomal drug- and steroid-metabolizing monooxygenases and tumor formation with more prolonged exposures.

The metabolic fate of PCBs in the liver has been studied extensively in efforts to determine the mechanisms involved in their hepatotoxicity. A number of PCB congeners are substrates for hepatic microsomal monooxygenases [10–13]. The highly chlorinated PCBs are resistant to metabolism but less chlorinated congeners are readily metabolized by hepatic enzymes. The hydroxylated metabolites identified *in vivo* and *in vitro* are apparently formed by the rearrangement of epoxide intermediates [10, 11, 14, 15]. Metabolism of some PCBs by the liver gives rise to reactive products which bind covalently to tissue macromolecules [10, 16–18] and are mutagenic in bacterial test systems [17]. In addition, some PCB metabolites are far more toxic than the parent compounds both *in vivo* and *in vitro* [15, 19]. Thus, many investigators believe that epoxide intermediates are responsible for the hepatotoxicity of PCBs, although other metabolites may also be involved [19, 20].

Although hepatic metabolism of PCBs has been studied extensively, the fate of PCBs in other tissues has received relatively little attention. Following the exposure of animals [6, 7, 21] or humans [22] to PCBs, very high levels have been found in the adrenal cortex. However, the disposition of PCBs within the adrenal cortex has not been investigated. Adrenal monooxygenases catalyze the oxidation of many foreign compounds including a variety of drugs and carcinogens [23]. In some cases, xenobiotic metabolism by adrenal enzymes results in the formation of reactive intermediates which cause both morphologic and functional changes in the gland [24–28]. The studies presented in this paper were done to determine if chlorinated biphenyls were substrates for adrenal microsomal monooxygenases and to compare adrenal and hepatic metabolism, both qualitatively and quantitatively. The regional localization of metabolism within the adrenal cortex was also examined. In these initial investigations, 4-chlorobiphenyl (4-CB), a compound whose hepatic metabolism has been well characterized [10, 12, 17], was used as a model substrate.

METHODS

Adult (900–1000 g) male English Short Hair guinea pigs obtained from the Camm Research Institute (Wayne, NJ) were used in all experiments. Animals were maintained under standardized con-

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ditions of light (6:00 a.m.–6:00 p.m.) and temperature (22°) on a diet of Wayne Guinea Pig Diet and water *ad lib*. Animals were decapitated between 8:00 and 9:00 a.m. Adrenals and livers were quickly removed and placed in cold 1.15% KCl containing 0.05 M Tris–HCl (pH 7.4). Tissues were homogenized, and microsomes and mitochondria were obtained as described previously [24, 28]. Microsomes were resuspended in KCl–Tris buffer and mitochrondria in 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4). In some experiments, adrenals were bisected longitudinally, and the dark-brown inner zone was gently dissected from the tan outer zone as described by Martin and Black [29]. Tissue from each zone was then homogenized, and microsomes from each were obtained by differential centrifugation.

Microsomal metabolism of 4-CB was studied by incubating adrenal or hepatic microsomes (equivalent to 5–40 mg wet weight tissue) in 1.15% KCl containing 0.05 M Tris–HCl (pH 7.4), 0.5 mM NADP, 10 mM sodium isocitrate, 0.15 units isocitrate dehydrogenase, 1.0 mg bovine serum albumin, and 1.8 mM $MgCl_2$ in a total volume of 1.0 ml. Mitochondrial incubations consisted of adrenal mitochondria (equivalent to 10–20 mg wet weight tissue) in 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4), 10 mM sodium isocitrate, 1.0 mg bovine serum albumin, and 1.8 mM $MgCl_2$ in a total volume of 1.0 ml. Metabolism was initiated by the addition of 4-CB in 10 μl acetone. After incubation, 4.0 μg biphenyl was added as an internal standard, and samples were extracted with 5 ml diethyl ether. The ether was evaporated under vacuum, and the residue was dissolved in HPLC grade methanol. An aliquot was injected into a Waters high pressure liquid chromatography system (Waters Associates, Milford, MA) equipped with a model 730 data module, a model 710B sample injector, and a model 721 programmable system controller. Compounds were separated using a radial-pak C_{18} cartridge which was eluted with a linear gradient of 85–100% methanol in water at a flow rate of 1.0 ml/min for 15 min. The eluent was monitored by absorbance at 254 nm. Standard curves for the extraction of authentic 4-CB, 4-chloro-4′-biphenylol (4′-OH-4-CB), and biphenyl from the incubation medium were obtained and stored in the data module. Thus, all values were corrected for any differences in recoveries from sample to sample.

Adrenal microsomal steroid 21-hydroxylase activity was assayed as the rate of 11β-hydroxyprogesterone conversion to corticosterone [30]. Benzo[*a*]pyrene hydroxylation by adrenal and hepatic microsomes was determined by the fluorometric method of Nebert and Gelboin [31]. Quinine sulfate was calibrated against authentic 3-hydroxybenzo[*a*]pyrene and routinely used as the fluorescence standard. Benzphetamine N-demethylation was assayed as the amount of formaldehyde formed using the method of Nash [32] as previously described [28]. For all enzyme assays, conditions were established to ensure linearity of product formation with respect to protein concentration and incubation time.

Mass spectral analyses of authentic 4-chloro-4′-biphenylol and the metabolites produced by microsomal incubations were done with a Finnigan model 4021 automated GC/MS equipped with an INCOS automatic data system. Samples were run at 20 eV by direct probe in the EI mode. Substrate-induced difference spectra were obtained using an Aminco DW-2a recording spectrophotometer as previously described [30]. Cytochrome P-450 was measured as the dithionite-reduced CO-complex as described by Omura and Sato [33]. Microsomal and mitochondrial protein concentrations were determined by the method of Lowry *et al.* [34] using bovine serum albumin as the standard.

RESULTS

Addition of 4-CB at concentrations of 50–500 μM to a suspension of adrenal microsomes (1.1 mg protein/ml) produced a typical type I difference spectrum (data not shown), indicative of substrate binding to cytochrome(s) P-450. A similar spectral change was produced in hepatic microsomal preparations. In both tissues, the magnitude of the spectral change was dependent upon the concentration of 4-CB. At saturating concentrations of 4-CB, the magnitude of the type I spectrum (per mg protein) was approximately twice as large with adrenal as with hepatic microsomes. 4-CB *in vitro* also inhibited several monooxygenase reactions in adrenal and hepatic microsomes (Table 1). Hepatic enzyme activities were decreased to a greater extent than adrenal

Table 1. Effects of 4-chlorobiphenyl (4-CB) *in vitro* on adrenal and hepatic microsomal drug and steroid metabolism*

Concn of 4-CB (M)	Enzyme activity (% of control)				
	Adrenal			Liver	
	Benzo[*a*]pyrene hydroxylase	Benzphetamine demethylase	Steroid 21-hydroxylase	Benzo[*a*]pyrene hydroxylase	Benzphetamine demethylase
1×10^{-5}	101 ± 4	98 ± 5	88 ± 5	89 ± 4	99 ± 6
1×10^{-4}	98 ± 6	93 ± 4	86 ± 2	68 ± 3†	89 ± 5
2.5×10^{-4}	97 ± 5	87 ± 5	80 ± 4†	59 ± 4†	81 ± 4†
5×10^{-4}	98 ± 5	83 ± 4†	77 ± 3†	57 ± 3†	77 ± 5†
1×10^{-3}	99 ± 4	78 ± 4†	82 ± 4†	55 ± 3†	75 ± 4†

* Values are expressed as mean percent of controls ± S.E., four to six determinations per value.
† $P < 0.05$ (vs controls).

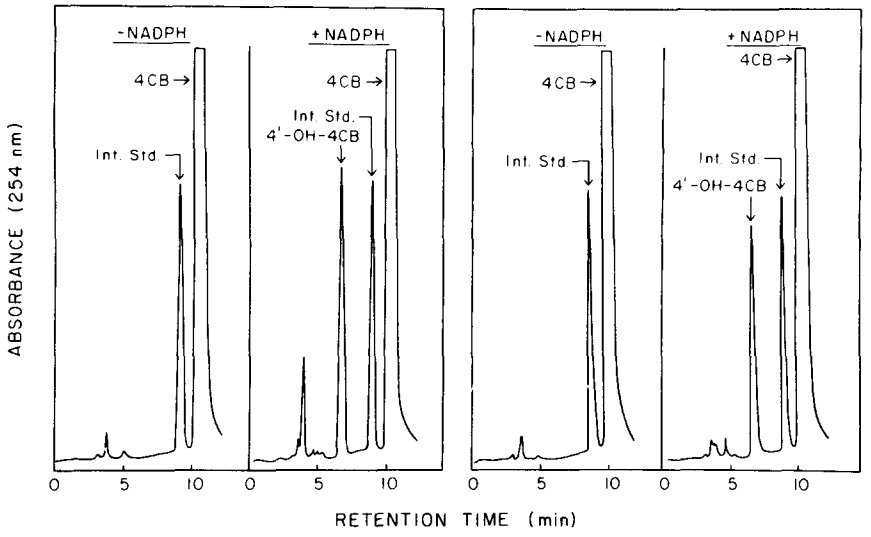

Fig. 1. HPLC analyses of 4-chlorobiphenyl (4-CB) metabolism by guinea pig hepatic (left panel) and adrenal (right panel) microsomal preparations. Hepatic (0.5 mg protein) or adrenal (0.2 mg protein) microsomes were incubated with 4-CB in the absence or presence of NADPH (0.5 mM) for 10 min, and metabolites were separated by HPLC as described in Methods. Peaks corresponding to 4-CB, 4-chloro-4′-biphenylol (4′-OH-4-CB), and the internal standard (int. std.), biphenyl, are noted. The effluent was monitored by absorbance at 254 nm.

activities. In the liver, benzo[*a*]pyrene hydroxylase and benzphetamine demethylase activities were decreased in a concentration–dependent manner by 4-CB. In contrast, adrenal benzo[*a*]pyrene hydroxylase activity was unaffected by 4-CB, but benzphetamine demethylase and steroid 21-hydroxylase activities were decreased significantly by the higher concentrations of 4-CB.

To determine if 4-CB was metabolized by adrenal enzymes, adrenal microsomal suspensions were incubated with 4-CB, and the products were analyzed by high pressure liquid chromatography (HPLC). In the absence of NADPH, metabolism of 4-CB by adrenal or hepatic microsomes was not demonstrable (Fig. 1). However, in the presence of NADPH or an NADPH-generating system, 4-CB was converted by both adrenal and hepatic microsomes to a major metabolite which eluted with a retention time identical to that of authentic 4-chloro-4′-biphenylol (4′-OH-4-CB). The identity of the metabolite was confirmed by mass spectrometry. Liver microsomes also produced smaller amounts of a more polar metabolite (Fig. 1) which is probably a dihydrodiol since its production was blocked by epoxide hydrolase inhibitors such as trichloropropene oxide (1.0 mM; TCPO) and cyclohexene oxide (10 mM). TCPO also caused a small but reproducible increase in the amount of 4′-OH-4-CB produced by hepatic microsomes but had no apparent effect on adrenal 4-CB metabolism (data not shown).

The amount of 4′-OH-4-CB produced by adrenal and hepatic microsomes was dependent upon both incubation time and microsomal protein concentration. The maximal rate of metabolite production was greater in adrenal than in hepatic microsomes (Table 2); the difference was approximately proportional to the difference in cytochrome P-450 concentrations in the two tissues (Table 2). However, the concentration of 4-CB needed for maximal rates of 4′-OH-4-CB production was far lower with hepatic than with adrenal microsomes (Fig. 2). Lineweaver–Burk plots revealed apparent K_m values of 41 and 269 μM for hepatic and adrenal microsomes respectively. Thus, substrate (4-CB) affinity was greater in hepatic than in adrenal microsomal preparations.

Cytochrome P-450-containing monooxygenases

Table 2. Adrenal and hepatic 4-chlorobiphenyl (4-CB) metabolism and cytochrome P-450 levels*

	Protein (mg/g tissue)	Cytochrome P-450 (nmoles/g tissue)	4-CB metabolism (nmoles/min × g tissue)
Hepatic microsomes	30.8 ± 1.9	30.9 ± 3.7	52.1 ± 4.0
Adrenal mitochondria	23.6 ± 2.2	11.8 ± 2.4	ND†
Adrenal microsomes			
Whole adrenal	41.7 ± 3.1	75.1 ± 8.3	133.4 ± 13.0
Inner zone	53.3 ± 3.4	111.6 ± 9.9	322.7 ± 86.9
Outer zone	31.4 ± 2.8	39.0 ± 3.6	70.3 ± 16.8

* Values are expressed as means ± S.E. of six to eight determinations.
† Not detectable.

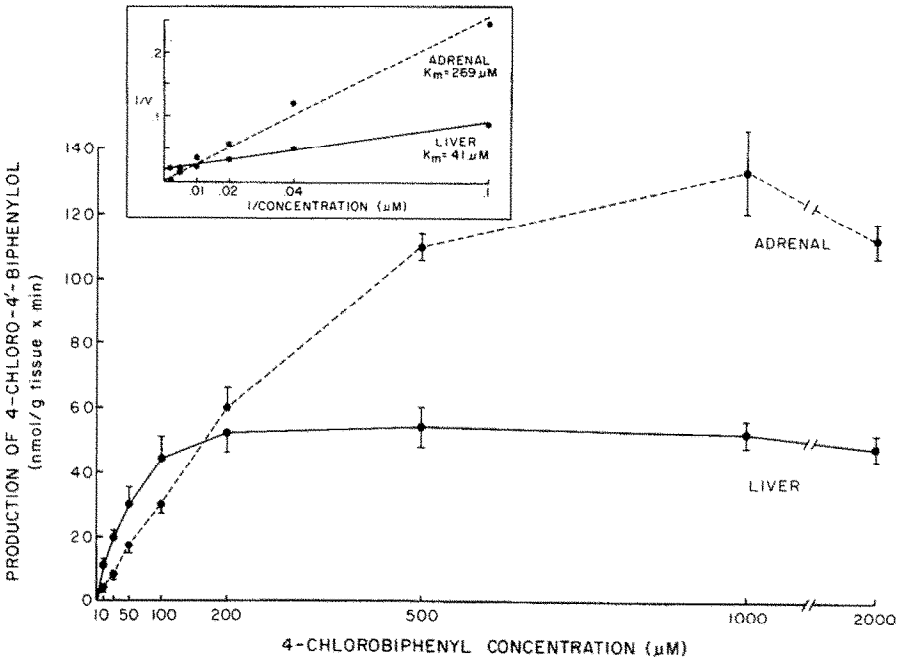

Fig. 2. Effects of 4-chlorobiphenyl (4-CB) concentrations on the rates of production of 4-chloro-4'-biphenylol by adrenal and hepatic microsomes. Adrenal (0.25 mg protein) or hepatic (0.5 mg protein) microsomes were incubated with various concentrations of 4-CB in the presence of NADPH (0.5 mM) for 5 min, and metabolites were separated and analyzed by HPLC as described in Methods.

are found in adrenal mitochondria as well as in microsomes [35], but 4-CB was metabolized only by microsomal preparations (Table 2). In addition, microsomal metabolism of 4-CB appeared to be localized primarily to the inner zone of the adrenal cortex (Table 2). After dissection of the adrenal cortices into the chromatically distinct inner (zona reticularis) and outer (zona fasciculata + zona glomerulosa) zones, as described by Martin and Black [29], microsomes were isolated from each and 4-CB metabolism was studied (Table 2). The rate of 4'-OH-4-CB production by microsomes from the inner zone was four to five times greater than that by outer zone preparations. The differences were attributable only in part to a higher concentration of cytochrome(s) P-450 in the inner zone (Table 2).

DISCUSSION

A wide variety of drugs and toxins are known to induce functional and morphologic changes in the adrenal cortex. In fact, the adrenal cortex appears to be the most sensitive of the endocrine organs to chemical-induced toxicities [36]. Although the role of metabolic activation in adrenal toxicity has not been studied extensively, there are several compounds whose effects on the adrenals are known to require metabolism. For example, the cytotoxic effects of 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane (*o*,*p*'-DDD) [25], 7,12-dimethylbenz[*a*]anthracene (DMBA) [27], and carbon tetrachloride (CCl_4) [26] on the adrenal cortex require local activation by cytochrome P-450-dependent monooxygenases. In addition, the inhibitory effects on adrenal steroidogenesis of spironolactone, a widely used antihypertensive agent, are apparently mediated by an adrenal metabolite of the drug [24]. Several reports [37–39] indicate that exposure to PCBs causes structural and functional changes in the adrenal cortex of experimental animals and humans, but it has yet to be established whether or not metabolic activation is required for the effects of PCBs on the adrenal cortex. However, reactive metabolites appear to be important in PCB-induced hepatotoxicity [10, 11, 15–18], and the data presented in this report indicate that chlorinated biphenyls can serve as substrates for adrenal microsomal monooxygenases. Thus, the potential for adrenal activation of such compounds exists, but further studies are needed before definitive conclusions can be reached.

The maximal rate of adrenal 4-chlorobiphenyl metabolism, when expressed per unit tissue weight, was more than double that of the liver. The difference is attributable, at least in part, to the higher adrenal microsomal protein and cytochrome P-450 concentrations. Various other xenobiotics are also rapidly metabolized by adrenal microsomal monooxygenases [23], in some cases specific activities far exceeding those for hepatic enzymes. The human fetal adrenal gland is one of the models in which high rates of xenobiotic metabolism have been demonstrated [40, 41]. The capacity to metabolize numerous foreign compounds may be an important factor contributing to the vulnerability of the adrenal cortex to chemical-induced lesions.

Qualitative as well as quantitative differences in adrenal and hepatic 4-CB metabolism were observed. The apparent affinity of 4-CB for hepatic

monooxygenases was far higher than for adrenal enzymes. The greater inhibition of hepatic than of adrenal monooxygenases by 4-CB *in vitro* is consistent with its higher affinity for hepatic enzymes. In addition, the maximal type I difference spectrum produced by 4-CB required far lower 4-CB concentrations in hepatic than in adrenal microsomes, indicating a higher affinity for hepatic cytochrome(s) P-450. Adrenal and hepatic microsomes also differed somewhat in the apparent pattern of 4-CB metabolism. Although the major metabolite in both tissues was 4-chloro-4'-biphenylol, liver microsomes also produced a more polar metabolite which was probably a dihydrodiol derivative. In the presence of epoxide hydrolase inhibitors, production of the metabolite was blocked and phenol production increased. Adrenal microsomes produced very little of the more polar metabolite and epoxide hydrolase inhibitors had no effects on adrenal 4-CB metabolism. We previously demonstrated that the activity of epoxide hydrolase, the enzyme which catalyzes the conversion of epoxides to dihydrodiols, is far greater in guinea pig hepatic than in adrenal microsomes [42]. As a result, hepatic metabolism of the carcinogen benzo[*a*]pyrene resulted in the production of proportionately more dihydrodiol metabolites than did adrenal metabolism. The differences in epoxide hydrolase activities may similarly account for the slightly different patterns of 4-CB metabolism by adrenal and hepatic microsomes. It is also possible that epoxide intermediates become rapidly bound to adrenal microsomal protein, resulting in very little dihydrodiol formation. That possibility is now being examined.

The cytochrome P-450-dependent monooxygenases which catalyze the hydroxylation reactions essential for steroidogenesis are found in both the microsomal and mitochondrial fractions of adrenocortical cells [35]. However, the hydroxylation of 4-CB occurred only in adrenocortical microsomes. Adrenal metabolism of most other xenobiotics is similarly limited to microsomal monooxygenases [23]. One notable exception is the adrenocorticolytic agent *o*, *p*-DDD, which is activated by adrenal mitochondrial enzymes but not metabolized by microsomal preparations [25].

Within the guinea pig adrenal gland, microsomal metabolism of foreign compounds appears to be highly localized to the inner cortical zone, the zona reticularis. We recently demonstrated that the activities of various xenobiotic-metabolizing enzymes were far greater in that zone than in the outer zones, the zona fasciculata and zona glomerulosa [43]. 4-CB metabolism also proceeded far more rapidly with microsomal preparations from the inner zone than those from the outer zone. The differences between the zones appear to be attributable, in part, to differences in cytochrome P-450 concentrations, but other factors also seem to be involved [43]. The higher rates of xenobiotic metabolism in the inner zone may account for the greater vulnerability of that region to the toxic effects of chemicals known to require metabolic activation [44]. In fact, we recently found that administration of CCl_4 to guinea pigs causes a selective necrosis of the zona reticularis of the adrenal cortex, the apparent site of CCl_4 activation [45]. Further studies are now needed to determine the site(s) of action of PCBs within the adrenal cortex and to establish whether the local production of reactive metabolites is involved in the adrenal toxicity of PCBs.

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