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In Vitro Metabolism of 4-Chlorobiphenyl by Control and Induced Rat Liver Microsomes†

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ABSTRACT: The in vitro metabolism, mechanism of metabolism, and macromolecular binding of a monochlorobiphenyl component of commercial polychlorinated biphenyls (PCB) have been investigated. 4-Chlorobiphenyl was metabolized by rat liver microsomes in the presence of NADPH to yield a major metabolite, 4′-chloro-4-biphenylol, and a number of minor metabolites. The metabolism of deuterium-labeled 4-chlorobiphenyl proceeded with the NIH shift of the isotope and no observed isotope effect thus indicating the intermediacy of an arene oxide. Noninduced rat liver microsomes mediated the covalent binding between the 4-chlorobiphenyl and 4′-chloro-4-biphenylol substrates and endogenous microsomal protein. Prior in vivo administration of a commercial PCB preparation, Aroclor 1248 (Monsanto Chemical Co., containing 48% by weight of chlorine), resulted in an induced microsomal preparation which significantly increased the substrate–protein binding. The effect of various inhibitors on protein binding was investigated. Aroclor 1248 induced microsomes mediated binding of 4-chlorobiphenyl to endogenous and exogenous nucleic acids, indicating a possible mechanism for the previously reported mutagenic action of this chlorobiphenyl. The spectral properties of Aroclor 1248 induced cytochrome P-450 were investigated and compared with the pentobarbital-induced cytochrome fraction.

Polychlorinated biphenyls (PCB)[1] are a class of chemically unreactive and thermally stable compounds that have found widespread industrial application as fire retardants, heat transfer fluids, plasticizers, and dielectric fluids. PCB were first identified in the environment during the late 1960's (Widmark, 1967) and since that time have been found to be among the most ubiquitous and persistent chemical pollutants in the global ecosystem (Hutzinger et al., 1974). The lipophilic nature of PCB results in their accumulation in tissues of species at higher trophic levels within terrestrial communities, and in aquatic species generally.

The biological effects of PCB are diverse and dependent on both the test animal and the dose levels; however, their hepatoxicity is probably the most widely recognized effect (Kimbrough, 1974). Recently it has been shown that female Sher-

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[1] Abbreviations used: PCB, polychlorinated biphenyls; TLC, thin-layer chromatography; TCPO, 1,2-epoxy-3,3,3-trichloropropane; BF, benzoflavone; PPO, 2,5-diphenyloxazole; PAH, polynuclear aromatic hydrocarbons.

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man strain rats fed on continued dietary supplements of PCB developed hepatocellular carcinomas and neoplastic nodules at a greater frequency than a control group (Kimbrough et al., 1975). Preliminary studies on workers believed to have been heavily exposed to PCB exhibited higher incidences of malignant melanomas in this group when compared with a nonexposed population (Bahn et al., 1976).

The lower chlorinated biphenyls currently in use in the electrical industries (e.g., Aroclor 1211) contain significant amounts of monochlorobiphenyl as well as polychlorobiphenyl components (Hutzinger et al., 1974). Previous studies have shown that as the degree of chlorination of biphenyl increases the metabolic rate decreases (Ghiasuddin et al., 1976; Mathew and Anderson, 1975) and the bacterial mutagenicity decreases (Wyndham et al., 1976). For these reasons the reactive 4-chlorobiphenyl isomer was chosen as a substrate to investigate metabolism and macromolecular binding in vitro.

4-Chlorobiphenyl and other PCB components are metabolized in vivo via arene oxide intermediates and binding to cellular macromolecules both in vivo and in vitro has been reported (Sundstrom et al., 1976; Wyndham et al., 1976). This study examines the routes of metabolism of 4-chlorobiphenyl, in vitro, in both a control and an induced rat liver microsomal enzyme preparation. This involves spectrophotometric characterization of the enzymes involved, studies of the mechanism of metabolism, and the extent of macromolecular binding, as well as evaluating the further metabolism and binding of the major product of 4-chlorobiphenyl oxidation, 4′-chloro-4-biphenylol.

Experimental Procedures

Materials. 4-Chlorobiphenyl (Eastman) was purified by thin-layer chromatography (TLC) and recrystallized from benzene; 4′-chloro-4-biphenylol was synthesized as described (Savoy and Abernethy, 1942). Tritiated 4-chlorobiphenyl and tritiated 4′-chloro-4-biphenylol were prepared by acid-catalyzed tritium exchange (New England Nuclear) and purified by TLC. The tritiated 4′-chloro-4-biphenylol was solvent exchanged with methanol prior to use. 4-Chloro[4′-^{2}H]biphenyl was prepared and purified (Safe et al., 1975b). 4-Chloro-[2′,3′,4′,5′,6′-$^2H_5$]biphenyl, at greater than 99% isotopic purity, was prepared by the addition of isoamyl nitrite (0.4 g) to 4-chloroaniline (0.8 g) in benzene-d_6 (10 g) (Cadogan, 1962) and the diazo coupling product purified by TLC. Substrate and metabolite purity and identity were determined by mass spectrometry using a Varian MAT CH-7 instrument. Aroclor 1248 was supplied by Monsanto Chemical Co., sodium pentobarbital by Diamond Laboratories. NADH, NADPH, poly(adenylic acid), poly(guanylic acid), poly(cytidylic acid), and poly(uridylic acid), and calf thymus DNA were supplied by Sigma Chemical Co. Denatured calf thymus DNA was prepared by heating to 100 °C for 2 min followed by rapid cooling. NCS tissue solubilizer is a product of Amersham/Searle and RNase T_1 was obtained from ICN. 1,2-Epoxy-3,3,3-trichloropropane (TCPO) and α-naphthoflavone (benzoflavone, BF) were supplied by Aldrich; SKF-525A was supplied by Smith Kline and French.

Induction of Microsomal Enzymes. Male Wistar rats weighing about 300 g were used for the study. Aroclor 1248 was dissolved in corn oil at 100 mg/mL and injected at 100 mg/kg intraperitoneally 4 days and again 2 days prior to sacrifice. Control animals received corn oil. Sodium pentobarbital in 10% alcohol, 40% propylene glycol in water (60 mg/mL) was injected in a linearly increasing dose from 50 mg/kg to 100 mg/kg for 4 consecutive days until 12 h prior to sacrifice. Animals were fed on Purina Rat Chow and water ad libitum and were on 12-h dark, 12-h light regimen. All treatments were received at 9 a.m., at the beginning of the light period.

Isolation of Microsomes. Induced and control animals were starved 24 h prior to sacrifice. Animals were decapitated and the livers pooled from three rats to yield a total of 24 g for each group. A crude microsome fraction, isolated as described (Pietropaolo and Weinstein, 1975) with certain modifications (Wyndham et al., 1976), was used for incubations in which binding to endogenous RNA was determined. For metabolism and binding to microsomal protein and exogenous nucleic acids, the crude microsomes were purified of adsorbed protein, and much of the endogenous RNA, by a method (Dallner et al., 1966) of rapid dilution in volumes of 0.15 M Tris (pH 8.0), 0.1 mM EDTA at 24 °C, followed by chilling to 4 °C and recentrifugation at 100 000g. The microsome fraction was adjusted to a protein concentration (determined by the method of Lowry et al., 1951) of 4 mg/mL.

Spectrophotometric Determinations. The concentration of cytochrome P-450 was determined for the microsomal fractions as described (Omura and Sato, 1964). Microsomes from pentobarbital and Aroclor 1248 induced rats were solubilized, fractionally precipitated with ammonium sulfate, and dialyzed according to a method previously described (Lu et al., 1972). The precipitate between 43% and 50% ammonium sulfate, calculated as if the solubilized microsomes did not contain glycerol (Green and Hughes, 1955), was used as the source of P-450. Temperature difference spectra were performed as described (Rein et al., 1977). Measurements were carried out on a Cary 118 spectrophotometer with water bath temperature control. The reference cell was maintained at 27 °C while the sample cell was cooled to 7 °C. The concentrations of P-450 and P-420 in the partially purified P-450 preparation were determined (Omura and Sato, 1964) as was the concentration of cytochrome b_5 (Raw and Mahler, 1959). When substrate was added, due to the limited solubility of 4-chlorobiphenyl, 150 μL of partially purified P-450 from pentobarbital or Aroclor 1248 induced microsomes dissolved in 20% v/v glycerol, 0.1 M Tris (pH 7.7), 0.1 mM dithiothreitol was mixed in the 0.1-cm spectrophotometer cell with 150 μL of 20% glycerol saturated with 4-chlorobiphenyl.

Microsomal Incubations. In a total volume of 3 mL, the incubation media contained 1.0 mL of 0.05 M Tris (pH 7.6), 0.3% Tween 80, with 0.1 mL of 0.01 M NADPH, 0.05 M Tris (pH 7.6), and 0.1 mL of [^{3}H]-4-chlorobiphenyl or [^{3}H]-4′-chloro-4-biphenylol in ethanol. Tritiated substrates were at specific activities of 0.15 mCi/mmol [3.0×10^{-7} mol (100 000 dpm)/0.1 mL] for metabolic incubations, or at 15.0 mCi/mmol [3.0×10^{-7} mol (1×10^7 dpm)/0.1 mL] for binding incubations. Binding to nucleic acids was repeated with [^{3}H]-4-chlorobiphenyl at a higher specific activity of 273 mCi/mmol. Exogenous nucleic acids (2.0 mg) were added in 0.8 mL of 0.05 M Tris (pH 7.6) or else the volume was made up with buffer alone. Incubations were started with 1.0 mL of microsomal suspension. Denatured microsomes were prepared by heating to 100 °C for 10 min. Metabolism of the deuterated substrates was carried out in bulk with 25× the incubation volume described. Incubation was at 37 °C open to atmospheric oxygen. For recovery of unmetabolized substrate and phenolic metabolites, incubations were stopped with 3 volumes of 2 M KOH and extracted with hexane directly from the incubation flask, with refluxing, followed by acidification to pH 2 to 3 and extraction with ether. Extracts were combined and analyzed either by chromatography on a 12-g Florisil column, eluting with 50 mL of hexane (4-chlorobiphenyl) then 25 mL of methanol (phenolic metabolites), or by TLC (MN-Keiselgel, UV 254, 0.8 mm) developed in chloroform, 1% acetic acid.

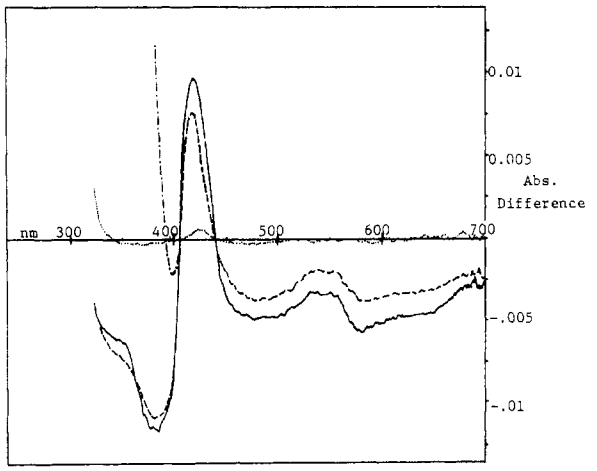

FIGURE 1: Temperature difference spectra in the visible range of partially purified cytochrome P-450 from sodium pentobarbital induced (—) and Aroclor 1248 induced (---) rat liver. The baseline (···) was recorded at ΔT = 0 °C, absolute temperature 23 °C, the difference spectra at ΔT = 20 °C, absolute temperatures 7 °C (reference) and 27 °C (sample). The difference spectrum of P-450 from Aroclor 1248 induced rats, in the presence of NADH, is superimposed on the original spectrum except at shorter wavelengths (····). The concentrations of cytochromes were: for the Aroclor induced preparation, P-450, 5.6 μM, P-420, 0.5 μM, b_5 1.8 μM; and for the pentobarbital induced preparation, P-450, 5.6 μM, P-420, 0.5 μM, b_5, 1.9 μM. The cell thickness was 0.1 cm.

Aliquots of isolated metabolites were counted in a toluene, PPO scintillation fluid with the Beckman LS-255 liquid scintillation counter.

Endogenous RNA and exogenous nucleic acids were recovered from incubation mixtures supplemented with 6% *p*-aminosalicylate, 1% NaCl, 0.1% sodium dodecyl sulfate by a 2 phase aqueous phenol, 0.1% sodium dodecyl sulfate extraction. The aqueous phase was extracted twice with phenol and the nucleic acids were precipitated with 0.1 volume of 2 M acetate (pH 4.7), 5 volumes of ethanol, followed by repeated precipitation from 2 mL of 0.015 M citrate (pH 7.0), 0.15 M NaCl to remove unbound substrate. Endogenous RNA was analyzed by sucrose density gradient (0.05 w/w to 0.25 w/w sucrose, 0.1 M Tris (pH 7.6), 0.1 M NaCl, 0.1 mM EDTA, centrifuged at 130 000*g* for 18 h at 4 °C) and characterized by alkaline and RNase T_1 hydrolysis as described (Wyndham et al., 1976). Recovery of nucleic acids was determined from the absorption maxima and corresponding extinction coefficients (Dunn and Hall, 1970) in the 260-nm region. In incubations containing microsomes alone, the progress of binding to microsomal protein and the effects of 1,2-epoxy-3,3,3-trichloropropane (TCPO), α-naphthoflavone (benzoflavone), and SKF-525A added to the incubations in 0.1 mL of ethanol were followed by precipitation with methanol followed by several washings with methanol (60 °C) and ethanol, water (3:2). All macromolecular fractions were counted by dissolving in 1.0 mL of NCS tissue solubilizer and counting an aliquot in a toluene, PPO scintillation fluid.

Results

Preparation of Microsomes. The cytochrome P-450 and cytochrome b_5 content of the microsomal enzyme preparation was monitored during purification of the crude rat liver homogenate. The 9000*g* supernatant from the control and Aroclor 1248 induced rats contained ca. 0.15 and 0.3 nmol of P-450/mg of protein, respectively. The crude microsome preparation following the first 100 000*g* centrifugation, which was used for determining the binding to endogenous ribosomal RNA, contained 0.6 and 1.2 nmol of P-450/mg of protein, respectively, while the 0.15 M Tris, pH 8.0, 0.1 mM EDTA washed microsomal preparation following the second 100 000*g* centrifugation contained 0.8 and 1.5 nm of P-450/mg of protein, respectively. Calibration of the spectrophotometer using a Holmium crystal with a characteristic absorption band at 453.7 nm indicated the position of the 450-nm absorption to be 448.5 and 449.5 nm for induced and control microsomes, respectively. The cytochrome b_5 content of the final microsomal preparation was 0.65 and 0.95 nmol of b_5/mg of protein for control and Aroclor induced microsomes.

Temperature Difference Spectra. Cytochrome P-450 exhibits weak absorption peaks in the visible region at about 568 and 538 nm and a strong absorption in the near-ultraviolet region at 418 nm (Levin et al., 1974). The positions of these absorptions, designated α, β, and γ or Soret maxima, respectively, are characteristic of intra-porphyrin $\pi \rightarrow \pi^*$ transitions of a low-spin Fe(III) hemoprotein (with a d^5 electronic configuration designated $^5t_{2g}$) (Smith and Williams, 1970). Several independent observations suggest however that a certain amount of high spin ($^3t_{2g}{}^2e_g$) character is associated with the P-450 Fe(III) hemoprotein. The Soret absorption maximum is at a low wavelength for a pure low spin hemoprotein, which should appear in the range of 420 to 425 nm (Smith and Williams, 1970) and with the addition of certain substrates the absorption at 380 to 400 nm in a simple absorption spectrum increases, indicating an increase in the high spin character (Rein et al., 1977).

Ligand splitting of the electronic energy levels of Fe(III) shows a transition at the point where the orbital splitting energy is equal to the spin pairing energy (Tanabe and Sugano, 1954), and a metal in a ligand field of about this strength will be in thermal equilibrium between the low-spin and high-spin states. Temperature difference spectroscopy then is a more sensitive probe of the spin state than simple absorption spectroscopy and, at a given ΔT, the amplitude and sign of absorptions indicate the degree of temperature dependence and, hence, the relative importance of the two spin states and the ground state of the hemoprotein. Figure 1 shows the temperature difference spectra of partially purified P-450 from Aroclor 1248 and pentobarbital induced rat liver microsomes. Pentobarbital was used because it is an inducer of cytochrome P-450 in contrast to the inducers, like polycyclic aromatics and Aroclor 248, of cytochrome P-448. There is an almost identical temperature dependence of the two cytochromes at equivalent concentration, and ΔT. There is a well-resolved minimum at 385 nm corresponding to a reduced absorption of the high-spin state, and a maximum at 420 nm corresponding to an increased absorption of the low-spin state, as the sample cell is cooled. From the signs of these absorptions a low-spin ground state is indicated, with a significant contribution of the high-spin state at physiological temperatures. There are less well-resolved maxima at 540 to 570 nm corresponding to the α and β low-spin absorptions, and broad minima at about 490, 590, and 640 nm corresponding to expected high-spin absorptions. Because the preparation contained cytochrome b_5 to about 20 to 30% of total heme, the contribution, if any, of this hemoprotein to the temperature difference spectrum was investigated by repeating the spectra in the presence of NADH (0.1 mM). Cytochrome b_5 is specifically reduced by NADH and the absorption maximum at 424 nm is used as the basis of quantifying the cytochrome. Any contribution of a b_5 Fe(III) spin equilibrium to the difference spectrum should be resolved by reducing the cytochrome to the Fe(II) oxidation state. The spectrum obtained for P-450 from Aroclor 1248 induced rats in the presence of NADH is identical with the original spectrum except for a strong positive absorption below 400 nm

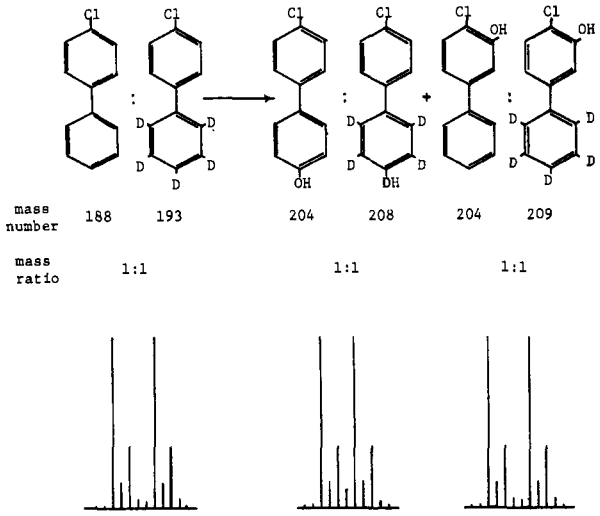

FIGURE 2: Mass spectra of the starting material and phenolic metabolites of Aroclor 1248 induced microsomal oxidation of 4-chlorobiphenyl and 4-chloro[2′,3′,4′,5′,6′-$^2H_5$]biphenyl.

which is a characteristic of Fe(III) spectra (b_5) and hence does not contribute to the original spectra.

The original intention of demonstrating the effect, if any, of binding of 4-chlorobiphenyl to P-450 was hampered by the limited solubility of the substrate. At saturation, 4-chlorobiphenyl did not significantly alter the temperature difference spectrum of either P-450 preparation, indicating a type II interaction (Rein et al., 1977); however, this cannot be shown definitely because of the tendency of the lipophilic substrate to bind nonspecifically to protein.

Mechanism of Metabolism. The in vitro metabolism of 4-chloro[4′-^{2}H]biphenyl with Aroclor 1248 induced rat liver microsomes gave the expected 4′-chloro-4-biphenylol metabolite as the major product. Mass spectral analysis indicated 79% retention of the deuterium atom and this is consistent with an arene oxide intermediate with a subsequent 1,2 migration or NIH shift of deuterium from the site of hydroxylation to the adjacent carbon atom (Jerina and Daly, 1974). Mass spectral analysis of a less polar minor monohydroxylated metabolite (M^+ 204) showed a deuterium content identical with that of the substrate (99% retention). Confirmation of this mechanistic pathway was obtained by incubation of a 1:1 mixture of 4-chlorobiphenyl and 4-chloro[2′,3′,4′,5′,6′-$^2H_5$]biphenyl with the induced microsomal enzyme preparation. The mass spectra of the substrate mixture and the phenolic metabolites are shown in Figure 2. The relative peak ratios of the molecular ions of the parahydroxylated metabolites and their hydrocarbon precursors were identical indicating no significant primary isotope effect in the hydroxylation on the deuterium substituted ring. The rate-limiting step in the reaction of arene oxides under physiological conditions involves heterolytic carbon-oxygen bond cleavage (Bruice and Bruice, 1976) and therefore the absence of an isotope effect in the metabolism of the 4-chlorobiphenyl/4-chloro[2′,3′,4′,5′,6′-$^2H_5$]biphenyl mixture is consistent with an arene oxide intermediate. The mass spectra of the minor monohydroxylated metabolite exhibited molecular ions of comparable intensity at *m/e* 204 and 209 confirming hydroxylation of the chlorine-substituted ring with no detectable steric effect due to the deuterium substitution. Examination of the mass spectrum of the corresponding methyl ether derivatives showed fragmentation with successive losses of CH_3 and CO from the molecular ion and no significant $M - CH_3Cl$ ions at *m/e* 168 and 173. Elimination of the elements of CH_3Cl from the molecular ion is highly characteristic for ortho-methoxylated chlorobiphenyls (Jansson and Sundstrom, 1974) and the absence of these ions supports the 4-chloro-3-biphenylol structure for this metabolite.

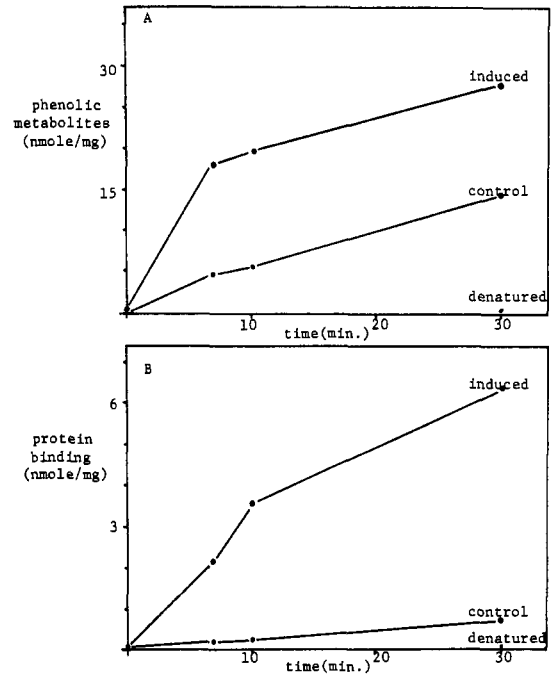

FIGURE 3: Metabolism and binding of [^{3}H]-4-chlorobiphenyl. (A) Progress curve for the formation of phenolic metabolites (mono-ol and diol) of 4-chlorobiphenyl. Induced and control microsomes incubated with substrate as described in Experimental Procedures. Denatured microsomes prepared by heating to 100 °C for 10 min. (B) Binding to microsomal protein during the oxidation of [^{3}H]-4-chlorobiphenyl.

In vitro studies with the induced rat liver microsomes did not result in sufficient dihydroxylated metabolite formation for mass spectrometric analysis; however, in vivo studies (rabbit) have revealed two diols with structures which would be expected if a second hydroxylation of the phenolic metabolites was taking place. The diols are 4′-chloro-3,4-biphenyldiol and 4′-chloro-3′,4-biphenyldiol. The TLC mobilities of these metabolites correspond to the radiochromatographic mobilities of the secondary metabolite(s) of 4′-chloro-4-biphenylol discussed in the next section.

Metabolism and Protein Binding. The metabolism of [^{3}H]-4-chlorobiphenyl was monitored by radio-TLC and the major radioactive products had R_f values which corresponded to the 4′-chloro-4-biphenylol and 4-chloro-3-biphenylol in vitro products identified by mass spectrometry (previous section) and to 4′-chloro-3,4-biphenyldiol and 4′-chloro-3′,4-biphenyldiol in vivo products identified by mass spectrometry and nuclear magnetic resonance spectroscopy (Safe et al., 1975b) and mass spectrometry (unpublished results), respectively. The approximate ratios of these products, in the order discussed, are 50:2:1 (diols) for Aroclor 1248 induced microsomal oxidation, and 20:1:1 (diols) for noninduced oxidation. Progress curves for the formation of metabolites with the noninduced and Aroclor 1248 induced rat liver microsomes are illustrated in Figure 3. The initial rate of 4-chlorobiphenyl hydroxylase activity for the noninduced system was 0.47 nmol min^{-1} mg^{-1} of protein and was linear for 30 min, whereas the initial rate of activity for the Aroclor 1248 induced enzymes was 2.6 nmol min^{-1} mg^{-1} of protein and showed a decrease in activity after less than 10 min of incubation. The effects of enzyme induction on binding to microsomal protein are even more dramatic as illustrated in Figure 3. The initial rate of binding of [^{3}H]-4-chlorobiphenyl to microsomal protein for the control enzymes

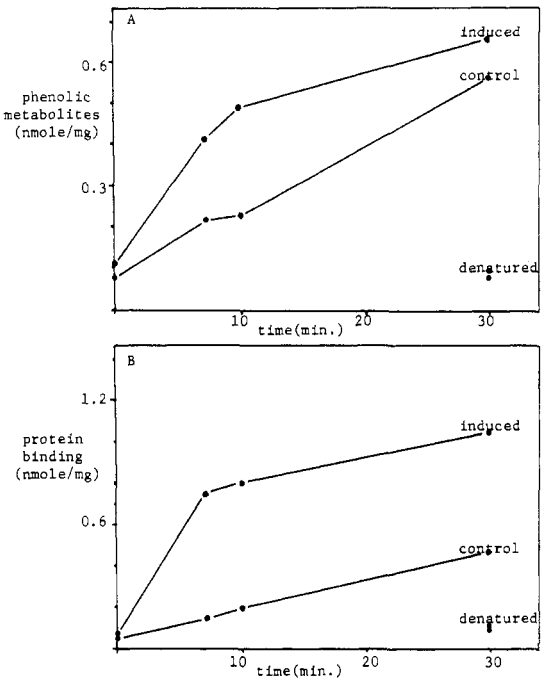

FIGURE 4: Metabolism and binding of [^{3}H]-4′-chloro-4-biphenylol. (A) Progress curve for the formation of diols of [^{3}H]-4′-chloro-4-biphenylol. (B) Binding to microsomal protein during the metabolism of [^{3}H]-4′-chloro-4-biphenylol.

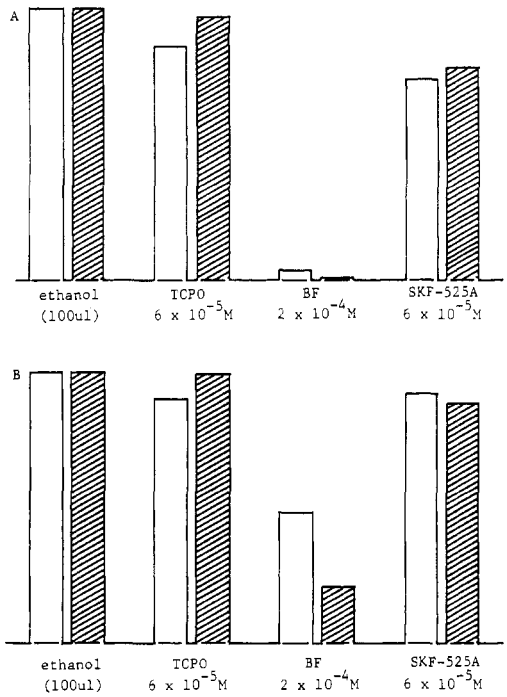

FIGURE 5: Effect of inhibitors on the binding to microsomal protein. (A) Binding of [^{3}H]-4-chlorobiphenyl in the presence of trichloropropylene oxide (TCPO), 7,8-benzoflavone (BF), and SKF-525A in relation to the binding observed in the presence of ethanol, the inhibitor solvent, for noninduced (open bars) and induced (shaded bars) microsomal incubations. (B) Binding of [^{3}H]-4′-chloro-4-biphenylol in the presence of inhibitors. Each observation represents the average of three incubations.

was 0.023 nmol min^{-1} mg^{-1} of protein, whereas the binding mediated by the induced microsomal enzymes was increased over 15-fold to 0.36 nmol min^{-1} mg^{-1} of protein.

A comparison of the in vitro metabolism of [^{3}H]-4-chloro-4-biphenylol with induced and control microsomes is shown in Figure 4. The initial rate of diol formation is 0.016 nmol min^{-1} mg^{-1} of protein for the noninduced microsomal enzymes and this is increased almost threefold to 0.044 nmol min^{-1} mg^{-1} of protein for the Aroclor 1248 induced microsomes. The in vitro formation of diol with the 4-chlorobiphenyl substrate is less than 0.05 nmol min^{-1} mg^{-1} of protein (with induced microsomes) and not surprisingly the 4′-chloro-4-biphenylol hydroxylase activity with the phenolic substrate is substantially lower (>50 times) than the 4-chlorobiphenyl hydroxylase enzyme activity. At this lower activity for the 4′-chloro-4-biphenylol substrate, however, the relative contribution of protein binding to the metabolism is on the order of ten times that of the parent hydrocarbon. Initial rates of protein binding for the 4′-chloro-4-biphenylol substrate are 0.015 and 0.07 nmol min^{-1} mg^{-1} for control and Aroclor 1248 induced microsomal enzymes.

The ratio of intial rate of protein binding/initial rate of hydroxylation for the 4-chlorobiphenyl substrate is 0.05 (noninduced) and 0.14 (induced) whereas the ratio for the 4′-chloro-4-biphenylol substrate is 0.95 (noninduced) and 1.6 (induced). In both Figure 3 and Figure 4, and in both the metabolic and protein binding progress curves (A and B), the control activities are linear for 30 min, whereas induced activities all show decreases at about 10 min after the start of incubation.

Effect of Inhibitors. Trichloropropylene oxide is an inhibitor of microsomal epoxide hydrase (Oesch et al., 1971) and has been shown to enhance the binding of polycyclic aromatic hydrocarbons to cellular macromolecules (Pietropaolo and Weinstein, 1975). In contrast, in the microsomal metabolism of both [^{3}H]-4-chlorobiphenyl and [^{3}H]-4′-chloro-4-biphenylol, trichloropropylene oxide significantly inhibits binding in the noninduced system and slightly inhibits binding in the induced system (Figure 5). Benzoflavone is an inhibitor of aryl hydrocarbon (polycyclic aromatic) hydroxylase of polycyclic aromatic induced rat microsomes, while it enhances the aryl hydrocarbon hydroxylase activity in noninduced rat liver microsomes (Wiebel et al., 1971). For noninduced and Aroclor 1248 induced microsome mediated binding of [^{3}H]-4-chlorobiphenyl to protein, benzoflavone is a powerful inhibitor (Figure 5A), virtually eliminating the binding to induced microsomes. The pattern is repeated for binding of [^{3}H]-4′-chloro-4-biphenylol, although the inhibition is not as complete. SKF-525A is thought to be a competitive substrate, showing irreversible type I binding in inhibiting microsomal oxidations (Anders, 1971). In noninduced and induced microsome mediated binding of [^{3}H]-4-chlorobiphenyl to protein, SKF-525A (6×10^{-5} M) at ca. one-half the concentration of the substrate (1×10^{4} M) inhibits binding to about 75% of control (Figure 5A). Binding of [^{3}H]-4′-chloro-4-biphenylol is inhibited to about 90% of control.

Binding to Nucleic Acids. Isolation of endogenous microsomal RNA from incubation mixtures of crude, Aroclor 1248 induced microsomes and [^{3}H]-4-chlorobiphenyl substrate reveals a microsome and time-dependent binding of substrate to this nucleic acid fraction. The radioactivity bound was rendered soluble by alkaline hydrolysis (0.5 M KOH at 37 °C for 18 h) or RNase T_1 hydrolysis (10 units of RNase T_1/OD260 RNA at 37 °C for 24 h) as indicated by precipitation with cold 10% trichloroacetic acid. Previous work had indicated extensive degradation of the ribosomal RNA during prolonged incubations (Wyndham et al., 1976) so that, for the analysis by sucrose density gradient, the rRNA isolated from a 10-min incubation was used (Figure 6). Because individual fractions contained very few counts, each was counted for 20 min with background subtract, and two gradients were analyzed. The

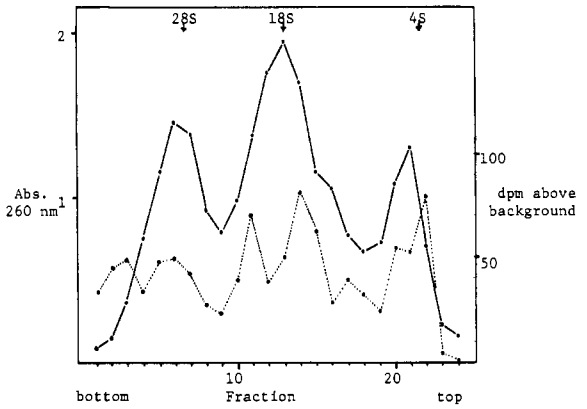

FIGURE 6: Sucrose density gradient analysis of endogenous ribosomal RNA recovered from induced, crude microsomes following incubation with [^{3}H]-4-chlorobiphenyl for 10 min. rRNA, 1.27 mg (from 27 mg of microsomal protein), with 940 dpm of bound [^{3}H]-4-chlorobiphenyl was layered over a 0.05 to 0.25 w/w sucrose density gradient and, following centrifugation (Experimental Procedures), was fractionated and the absorbance at 260 nm (●—●) and the radioactivity above background (●----●) were determined.

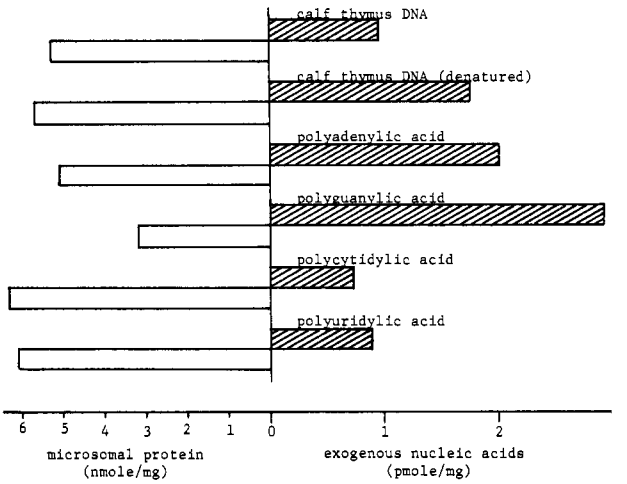

FIGURE 7: Binding of [^{3}H]-4-chlorobiphenyl to exogenous nucleic acids and microsomal protein in 60-s incubations supplemented with a series of natural and synthetic nucleic acids.

pattern of binding was identical for the second incubation, including the peak in fractions 2 and 3 and the two peaks associated with the 18S rRNA material.

Incubation of [^{3}H]-4-chlorobiphenyl with induced microsomal enzymes and exogenous calf thymus DNA showed a time-dependent uptake of radioactivity into the DNA with maximum uptake observed after 60 min. Comparable binding experiments with a series of natural and synthetic nucleic acids are summarized in Figure 7. Denatured calf thymus DNA is more readily alkylated than the natural material and the synthetic homopolynucleotides poly(A) and poly(G) are better targets than the poly(C) or poly(U) analogues. Binding to microsomal protein in these identical incubations is comparable to that observed following 60-min incubation without exogenous nucleic acids, except with poly(G) and to some extent poly(A). In these incubations binding to microsomal protein is inhibited.

Discussion

Commercial PCB and isomeric chlorobiphenyls are readily metabolized by diverse animal and microbial systems to yield hydroxylated products (Sundstrom et al., 1976; Hutzinger et al., 1972; Gardner et al., 1973), and not surprisingly both the parent hydrocarbon and the metabolites have been identified in wildlife samples (Jansson et al., 1975). The metabolism of 2,2′,5,5′-tetrachlorobiphenyl by the rat yielded several phenolic metabolites including 3,4-dihydro-2,2′-tetrachloro-3,4-biphenyldiol (Gardner et al., 1973). Subsequent in vivo and in vitro studies have shown that the metabolism of several isomers is accompanied by the NIH shift of deuterium or chlorine (Safe et al., 1975b, 1976b; Wyndham et al., 1976) and the results are consistent with the formation of an arene oxide as a metabolic intermediate (Jerina and Daly, 1974). It has been shown that many chemical carcinogens are also metabolized via a microsomal mixed function oxidase system to give reactive intermediates which subsequently rearrange to give metabolites or covalent adducts with critical cellular macromolecules. The carcinogenic polynuclear aromatic hydrocarbons (PAH) are prime examples of this class of chemicals in which their corresponding arene oxides represent the physiologically active cellular alkylating agent or ultimate carcinogen (Miller and Miller, 1974; Heidelberger, 1975).

This parallel in the metabolic mechanism between a number of chlorobiphenyls and other, well investigated, toxic aromatic hydrocarbons suggested a detailed study of a reactive chlorobiphenyl was in order. The parallel was lent further support with observation of the mutagenicity of 4-chlorobiphenyl, and of Aroclor 1221 which contains ca. 50% monochlorobiphenyl as its major component, in a bacterial/microsome supplemented frameshift mutagenicity assay (Wyndham et al., 1976).

Because it had already been shown (Wyndham et al., 1976) that binding to endogenous RNA in a noninduced rabbit microsomal preparation was very low, it was decided that parallel studies with noninduced and Aroclor 1248 induced rat (a species strongly inducible by hydrocarbons) microsomes would be carried out. The pretreatment described increased the concentration of cytochrome P-450 twofold in all preparations. This obviously cannot be the limiting factor in the microsomal metabolism of 4-chlorobiphenyl since all induced microsomal rates were more than twice the control rates.

The observation that both Aroclor 1248 induced and pentobarbital induced cytochrome P-450 (or P-448.5 and P-449.5, respectively) show temperature dependent, low spin–high spin equilibria can be discussed in terms of the role of P-450 in the oxidation of substrates like 4-chlorobiphenyl. At some point in the reaction cycle oxygen becomes bound to P-450. In the high-spin complex of Fe(III), the expanded bonding radius of the metal (with 2 electrons in antibonding e_g orbitals) results in the metal lying out of the plane of the heme with a weaker ligand field and greater bonding distance to at least one of the nonheme ligands (Hoard, 1971; Rein et al., 1977; Perutz, 1970). This weak bonding position may facilitate the reduction to a five-coordinated high-spin, Fe(II) complex required for binding oxygen during the catalytic cycle (Rein et al., 1977).

The hydroxylase activity of the induced and control enzymes was assayed using the hydrocarbon, 4-chlorobiphenyl, and its major metabolite, 4′-chloro-4-biphenylol, as substrates. The results indicated that the hydrocarbon is the most reactive substrate and that the Aroclor 1248 induced enzymes have increased hydroxylase activity for both the hydrocarbon and phenol. Incubation of 4-chloro-4′-[^{2}H]biphenyl gave the phenol 4′-chloro-4-biphenylol as the major product with 79% retention of deuterium. This confirmed the previous metabolic studies with rabbits in which the NIH shift of deuterium was also observed (Wyndham et al., 1976; Safe et al., 1975b). The metabolism of equimolar mixtures of 4-chlorobiphenyl and

4-chloro[2′,3′,4′,5′,6′-$^2H_5$]biphenyl showed the formation of the 4-hydroxy metabolite without any apparent isotope effect. This was also consistent with an arene oxide intermediate since it has been shown that rearrangement into a phenol does not involve carbon–hydrogen (or deuterium) bond cleavage in the rate-determining step (Jerina and Daly, 1974; Tomaszewski et al., 1975). Mass spectral analysis also suggested that the likely structure of a minor monohydroxylated product (ca. 3–5% of the metabolite yield) was 4-chloro-3-biphenylol in which hydroxylation of the chloro-substituted ring had occurred. Although the substituted ring is more deactivated this type of minor metabolic product is not without precedent in PCB metabolism (Tulp et al., 1976).

The incorporation of the radiolabeled substrate and microsomal protein was also determined in these experiments. The control and Aroclor 1248 induced microsomes mediated binding between the protein fraction and both 4-chlorobiphenyl and 4′-chloro-4-biphenylol. The protein binding exhibited by the latter substrate was significantly lower than that observed for 4-chlorobiphenyl; however, for both induced and control enzyme systems the ratio of the initial rate of protein binding/initial metabolism rate was higher for 4′-chloro-4-biphenylol. Clearly this metabolite does not exhibit the remarkable macromolecular binding and carcinogenicity noted for (±)7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, a metabolite of the procarcinogen benzo[*a*]pyrene (Remsen et al., 1977; Weinstein et al., 1976; Huberman et al., 1976; Thompson et al., 1976). The data, however, do indicate that microsomal enzyme induced metabolism tends to result in a relatively higher proportion of protein binding of the phenol when compared with the parent hydrocarbon. Previous studies have shown that 4′-chloro-4-biphenylol is a substrate for tyrosinase-like monooxygenase enzymes (Safe et al., 1976a) giving the 4′-chloro-3,4-biphenyldiol metabolite; however, there is no evidence that tyrosinase-like enzymes promote covalent substrate–macromolecular adducts. The binding of 4′-chloro-4-biphenylol to microsomal protein and the inhibition of binding by benzoflavone suggest that secondary metabolism of the metabolite 4′-chloro-4-biphenylol involves the cytochrome P-450 monooxygenase enzymes, and the intermediacy of an arene oxide possibly in the chloro-substituted ring. The lack of enhancement of protein binding in the presence of trichloropropylene oxide, an arene oxide hydratase inhibitor, indicates that this enzyme is not effective in the hydration of the active intermediate of either 4-chlorobiphenyl or 4′-chloro-4-biphenylol oxidation. The slight inhibition of binding of both substrates in noninduced microsomal incubations may be due to a competitive interaction. SKF-525A, a competitive inhibitor, is more effective in inhibiting the binding of [^{3}H]-4-chlorobiphenyl, and in fact in all three inhibitors, the binding of the parent hydrocarbon is reduced to a greater relative amount than the binding of the phenol.

The macromolecular binding of the more active substrate, 4-chlorobiphenyl, with endogenous and exogenous nucleic acids was also investigated since this adduct formation is presumed to be the most important causative factor in the action of chemical carcinogens (Miller and Miller, 1974; Heidelberger, 1975). The binding of [^{3}H]-4-chlorobiphenyl to endogenous microsomal RNA is nonspecific. The radioactivity is associated with the 28S, 18S, and 4S material. This contrasts to the binding of benzo[*a*]pyrene and 4-chlorobiphenyl to a lower molecular weight endogenous microsomal RNA component (Wyndham et al., 1976; Pietropaolo and Weinstein, 1975).

Incubation of 4-chlorobiphenyl and the induced microsomal enzyme fraction with exogenous nucleic acids such as calf thymus DNA, denatured calf thymus DNA, poly(A), poly(G), poly(C), and poly(U) also resulted in the formation of nucleic acid–4-chlorobiphenyl adducts. Due to the limited amount of alkylation observed, it was not possible to determine the nature of the adducts formed; however, since poly(A) and poly(G) have a greater nucleophilic character it is conceivable that their respective amino substituents may be involved. The nature of the inhibition of binding to protein by poly(G) and to a lesser extent poly(A) is not known; however, it has been suggested (Murray et al., 1976) that in the presence of the guanine nucleotide the conversion of benzo[*a*]pyrene 4,5-oxide to a phenolic metabolite is enhanced. This may be an underlying factor in the observed effect. No detectable binding to nucleic acids was observed in the noninduced system; however, this is not surprising considering the lower metabolic activity.

These results show a parallel between 4-chlorobiphenyl (a component of PCB) and many known chemical carcinogens with respect to their metabolism, mechanism of metabolism, and their subsequent interaction with cellular macromolecules, and the data complement the known bacterial mutagenicity of 4-chlorobiphenyl (Wyndham et al., 1976). Additional in vivo and in vitro studies are required to further define the potential health hazards of the halogenated biphenyl pollutants.

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Effect of Alterations in the Amphipathic Microenvironment on the Conformational Stability of Bovine Opsin. 1. Mechanism of Solubilization of Disk Membranes by the Nonionic Detergent, Octyl Glucoside†

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ABSTRACT: The mechanism of solubilization of the bovine retinal rod outer segment disk membrane by the nonionic detergent, octyl β-D-glucoside, was investigated. Saturation of the membrane by detergent occurred at a level of 1.4 mol of detergent bound per mol of disk membrane phospholipid. Increasing the detergent concentration beyond the level of membrane saturation resulted in the release of rhodopsin–lipid–detergent complexes containing 25–50 mol of phospholipid and 270 mol of octyl glucoside per mol of rhodopsin. The composition of the phospholipids in these initially solubilized complexes was identical (±5%) with that of the native disk membrane, suggesting that rhodopsin does not interact preferentially with any one of the three major classes of phospholipids present in the disk membrane. Direct binding measurements showed that the amount of phospholipid contained in the solubilized rhodopsin–lipid–detergent complexes ranged from 25 mol/mol of rhodopsin in the initial stage of solubilization to 50 mol/mol of rhodopsin at the point of complete disk membrane solubilization. At detergent concentrations above the fully solubilizing level, the amount of rhodopsin-associated phospholipid decreased with increasing detergent concentration. The distribution of phospholipid in the system was as expected for partitioning of phospholipid between the rhodopsin–lipid–detergent complexes and rhodopsin-free lipid–detergent micelles.

The integral membrane proteins, by definition, exist in close association with phospholipid and derive many of their functional and structural properties from this association. This is evidenced by the fact that the activities of membrane-bound enzymes are usually lost when the phospholipid is removed (Helenius and Simons, 1975; Tanford and Reynolds, 1976). In order to better understand the way in which phospholipid acts to stabilize the native conformation of integral membrane proteins, we have measured the changes in the conformational stability of bovine opsin which occur when the native pro-

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