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Effect of PCB and DES on Rat Monoamine Oxidase, Acetylcholinesterase, Testosterone, and Estradiol Ontogeny

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Diethylstilbestrol (DES) and polychlorinated biphenyl (PCB) have been documented as potentially hazardous environmental agents. *In utero* exposure to DES produces human vaginal adenocarcinoma (Herbst et al. 1971; Greenwald et al. 1971), male reproductive tract lesions in mice (McLachlan et al. 1975), and has been correlated with personality changes in human males (Reinisch 1977). PCB (Kanechlor) was found to be the major toxin in the "Yusho" rice oil poisoning in Japan in 1968 (Kuratsune et al. 1975). Other investigators have shown in rats that PCB (Arochlor) causes liver adenofibrosis (Kimbrough et al. 1973), thyroid dysfunction (Bastomsky 1974), atypical mitochondria, and dilation of both smooth and rough endoplasmic reticulum (Cordle et al. 1978). Matthews et al. (1978) also reported that 4,4' chlorinated biphenyl was the most potent inducer of monooxygenases, irrespective of chlorination at other sites. Although these compounds have been studied extensively in mammals, there is a paucity of data examining their effects when non-fetotoxic amounts are administered chronically and orally during gestation.

The present study is part of a larger effort designed to establish a protocol for testing the developmental effects of xenobiotics such as DES and PCB. Levels of acetylcholinesterase (AChE) were measured as an indicator of the integrity of nerve transmission in the central nervous system. Monoamine oxidase (MAO) is a marker for the outer mitochondrial membrane (Schnaitman et al. 1967) and is an important amine metabolizing enzyme. Testosterone and estradiol are important sex steroids in mammals, and effects upon levels of the two hormones may signal anomalies in development of sex characteristics.

MATERIALS & METHODS

Adult (150-200 g) virgin female Sprague-Dawley rats were mated to males of the same strain. Conception (day zero) was determined by the presence of a mucous plug. All animals were maintained on a 12L:12D schedule. Beginning with day six of gestation, each dam was dosed each of 13 consecutive days (6-18) with either DES or PCB. DES and a pure sample of a 3,3', 4,4' tetrachloro biphenyl congener (4-CB) were obtained from Sigma Chemical Company and the National Institute of Environmental Health Sciences, respectively. The compounds were administered by oral gavage in corn oil at 10 µg DES/kg/d, and 3 mg 4-CB/kg/d. Controls dams were given similar amounts of corn oil only.

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On days 15, 17, 19, and 21 of gestation, dams were sacrificed by cervical dislocation and fetuses removed. Pups were sacrificed in a similar manner on days 1, 5, 10, 21, 35, and 56 postnatal development. Brains and livers from fetuses and/or pups were maintained at 0°C and homogenized in 0.03 M TRIS buffer (pH=8.0). Enzyme activities in fetal homogenates are reported as litter averages, based on two separate determinations of tissues pooled from each uterine horn. Female and male samples from each litter were assayed separately in neonates. The final wet weight concentrations of the tissue homogenates at all ages were 150 mg/ml for whole brain and 100 mg/ml for liver.

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AChE activity was measured using a modification of the spectrophotometric technique of Ellman et al. (1961) (assay kits purchased from Boehringer-Mannheim Corporation). Each enzyme assay was measured in triplicate for three minutes at room temperature measuring the increased absorbance due to thionitrobenzoate at 405 nm. Fifty microliters of brain homogenate was used for determinations of activity in fetuses and one-day-old pups, and 20 µL of homogenate was used for measurements of AChE activity in 5 56-day old pups. There was a linear response in activity of the enzyme throughout that concentration range. The unit of AChE activity is defined as nmol acetylthiocholine hydrolyzed/min/mg protein.

A modification of the microradiometric assay developed by Urry et al. (1972) for MAO in rat seminiferous tubules was used to measure MAO activity in brain and liver homogenates. Specific changes in the assay procedure were the use of [side chain-2-^{14}C] tyramine (Amersham Searle Corporation), which is deaminated by both A and B isozymes of MAO, as substrate rather than [^{14}C] 5-hydroxy tryptamine, a substrate sensitive only to MAO A (Tipton et al. 1976). Triplicates of each homogenate were assayed for 10 rather than 30 minutes. Also, the tubes used for the second extraction of product were not centrifuged. In comparison to 5-hydroxy tryptamine, tyramine produced blanks of ca. 2500 dpm and the assay produced a linear response through ca. 45,000 dpm/50 µL final product. MAO specific activity is defined as nmol tyramine deaminated/min/mg protein.

Bradford's technique (1976) was used to measure protein.

Blood was obtained by cardiac puncture from 35 day (prepubertal) and 70 day (postpubertal) pups. Stages of estrus were not determined in postpubertal females. All animals were sacrificed by cervical dislocation. Serum was collected from clotted blood samples with the aid of a table-top centrifuge fitted with a swinging bucket rotor. The serum was stored at 0°C until assayed for testosterone and estradiol by radioimmunoassay (RIA). Reagents were purchased from Radioassay Systems Laboratories, Carson, California, and methods used are outlined by Abraham et al. (1977). Serum samples of ca. 1 mL volume were extracted with 3:2 (v:v) ethyl acetate:hexane, and the extracts were dried. Dried extract was solvated with isooctane (2,2,4-trimethylpentane) and then chromatographed on microcelite columns to isolate pure fractions of testosterone and estradiol from the same serum sample. The purified steroids were dried and then solvated with buffer solution and assayed. Steroid concentrations were calculated by predictions from a logit-log least squares regression of the binding curve.

Developmental profiles of enzyme activities were analyzed statistically by the linear model approach [Y(ijk) = Age (i) + Treatment (j) + TA + ε(ijk)] available in the computer program *Rummage* (Analysis of Variance with Expected Mean Squares, Dr. Melvin W.Carter, Statistics Department, Brigham Young University, Provo, Utah.) Enzyme activities for day 19 of gestation were used as the standard of comparison for other developmental points. Hormone data were analyzed via a one-way analysis of variance.

RESULTS & DISCUSSION

The biochemical effects of DES and 4-CB which we have observed are best assessed in conjunction with their biological effects. Both DES and 4-CB produced significant numbers of resorptions by day 19 of gestation (control = 8%, DES = 10-100%,4-CB = 14%) (Simmons et al. 1984). Neonatal mortality (stillborn plus pups dead one day after birth) was also significantly elevated by DES, and was 44% higher than controls in 4-CB-treated litters (Simmons et al. 1984). DES administration did not result in a dose-response increase in gross malformations; however, 4-CB treatment produced intestinal hemorrhage and blood in the amniotic fluid of 19 day fetuses (Wardell et al. 1982).

The development of AChE in brain tissue was not affected by DES or 4-CB. Statistical analysis of the overall developmental pattern revealed no significant differences ($\alpha = 0.05$) between control and DES-treated (Figure 1A) or 4-CB-treated (Figure 1B) animals. Beginning with day 15 of gestation, specific activity doubles every two days until birth. The postnatal level continues to rise at about the same rate until a plateau is reached at day 35. The shape of this profile is the same as that reported by Maletta et al. (1967). Analysis of the specific activity levels in males and females showed no statistical difference.

MAO development in brain was not altered by administration of DES (Figure 2A), but 4-CB depressed specific activity overall by 28% ($p < 0.05$) (Figure 2B). Point comparisons of the developmental profile in control and 4-CB-dosed animals indicated significant differences ($p < 0.10$) for one-day-old and 21-day-old pups. The developmental profile for MAO in control animals contains an apparent discontinuity (activity spike) at birth. A similar discontinuity in 4-CB-dosed animals (Figure 2B) occurred about 48 hours later. In controls, the activity of MAO at birth was about 35% that of 56-day-old pups (adults) and agrees with other published data (Gripois 1975). There were no significant differences in MAO activity between female and male pups.

The developmental pattern of MAO in liver was not altered significantly by DES (Figure 3A). However, 4-CB reduced MAO activity in liver over the whole curve by 42% ($p < 0.005$) (Figure 3B). The activity spike in control liver was statistically significant and occurred about 48 hours late in 4-CB-dosed animals. Liver MAO levels in male and female pups was the same.

Compared with control levels, male serum testosterone was depressed ca. 73% ($p < 0.01$) in 35-day DES-dosed and 4-CB-dosed males (Table 1). By day 70, testosterone levels in DES-treated males had returned to control levels, but testosterone in 4-CB-dosed males remained lower than control by ca. 40% ($p < 0.05$). Female serum testosterone, and male and female serum estradiol were not affected by either compound. Average recoveries of testosterone and estradiol for the RIA were 70% and 50% respectively.

Of the two xenobiotics, 4-CB has a record of affecting neurological development. Lucier et al. (1977) reported that mice administered 4-CB prenatally (32 mg/kg/d) exhibited a behavioral deficit characterized as 'Waltzing Syndrome'. The same effect has not been observed in the rat. Our data indicate that the pattern of AChE development in the rat in not altered by 4-CB treatment. Whatever subtle effects on the ontogeny of the nervous system this compound may produce must be directed at some other molecular target.

In addition to the reduction by 4-CB in MAO activity, there is some interest in the normal developmental profile of the rat enzyme revealed in our data. Significant qualitative

Table 1. Sex hormone levels in serum from Sprague-Dawley rats

Age (days)	Sex	Testosterone (ng/ml) (n)	Control	(n)	DES	(n)	4-CB	Estradiol (ng/ml) (n)	Control	(n)	DES	(n)	4-CB
+35	M	(8)	2.271[a] ±1.130	(8)	0.609[b] ±0.349	(5)	0.636[b] ±0.309	(8)	0.098 ±0.093	(9)	0.132 ±0.147	(5)	0.066 ±0.093
	F	(9)	0.160 ±0.111	(8)	0.229 ±0.258	(4)	0.327 ±0.278	(9)	0.087 ±0.024	(8)	0.071 ±0.047	(4)	0.047 ±0.042
+70	M	(11)	4.180 ±2.190	(14)	4.985 ±3.295	(4)	2.558[c] ±1.186	(11)	0.052 ±0.029	(14)	0.064 ±0.037	(4)	0.055 ±0.010
	F	(10)	0.243 ±0.128	(13)	0.308 ±0.162	(5)	0.393 ±0.367	(10)	0.071 ±0.033	(13)	0.084 ±0.033	(5)	0.079 ±0.073

[a]mean ± standard deviation

[b]$P<0.01$

[c]$P<0.052$

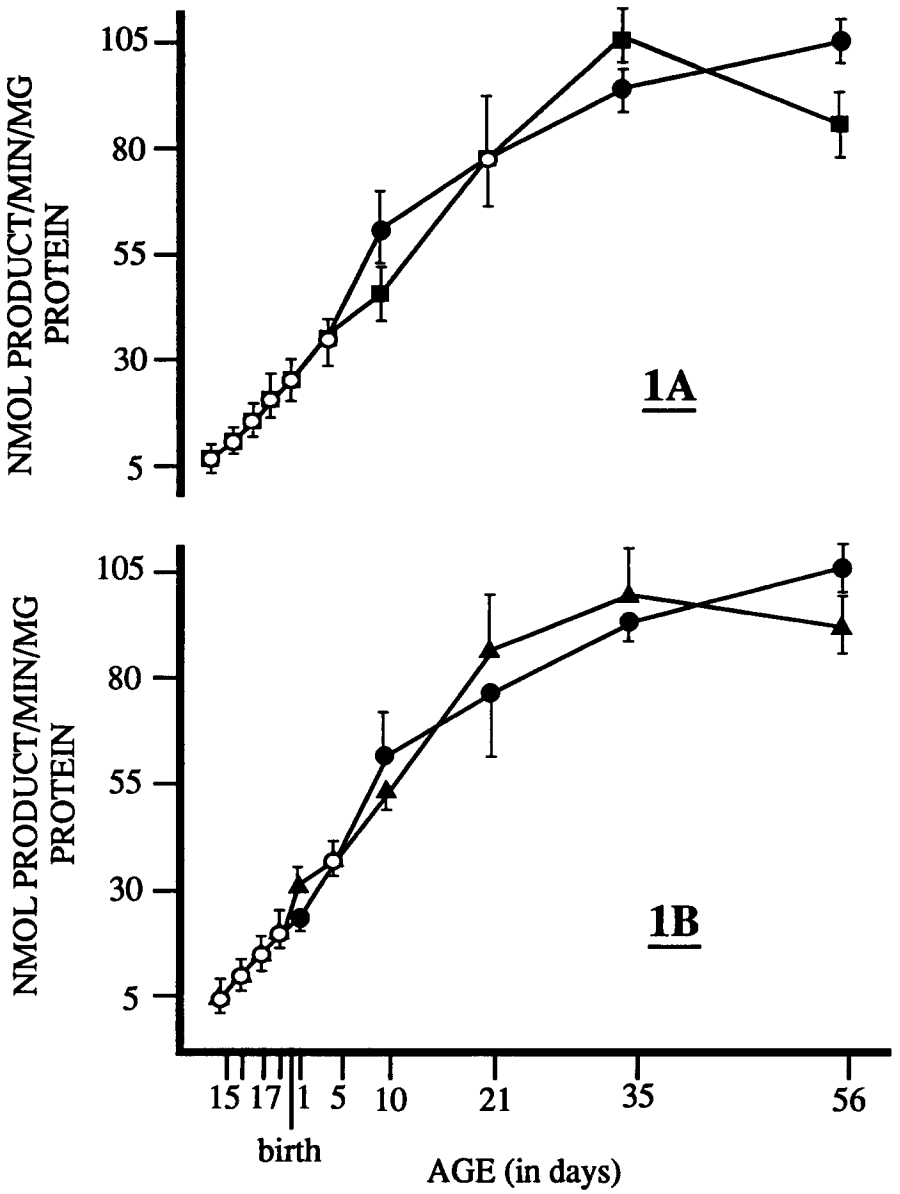

Figure 1. Prenatal and postnatal development of whole brain AChE Sprague-Dawley rats. Points are average ± s.e.m. for all litters in each age and treatment group. Figure 1A = comparison of control group (●) and DES group (■). Figure 1B = comparison of control group (●) and 4-CB group (▲).

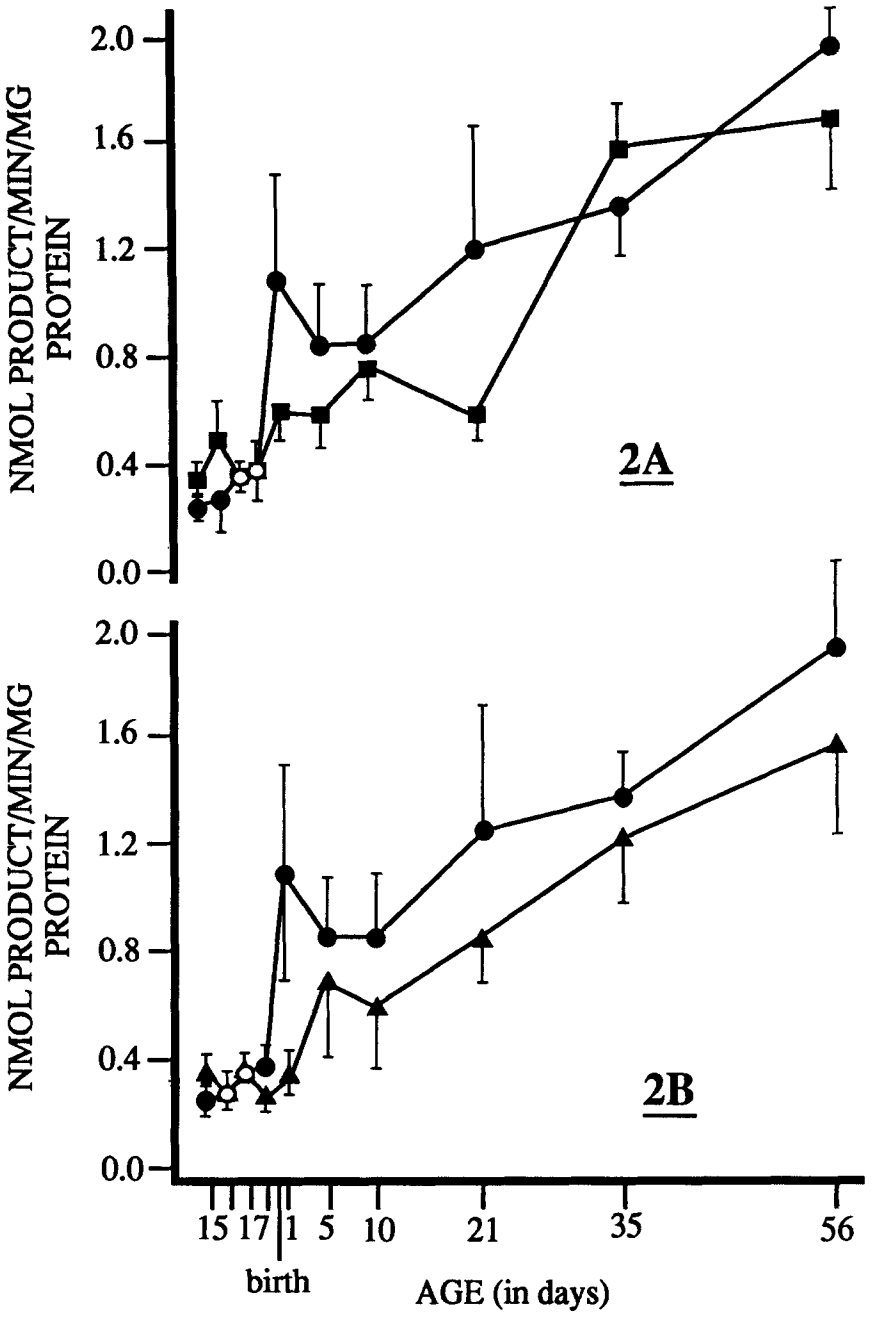

Figure 2. Prenatal and postnatal development of MAO in whole brain of Sprague-Dawley rats. Points are average ± s.e.m. for all litters in each age and treatment group. Figure 2A = comparison of control group (●) and DES group (■). Figure 2B = comparison of control group (●) and 4-CB group (▲) (significance between curves: $p < 0.05$).

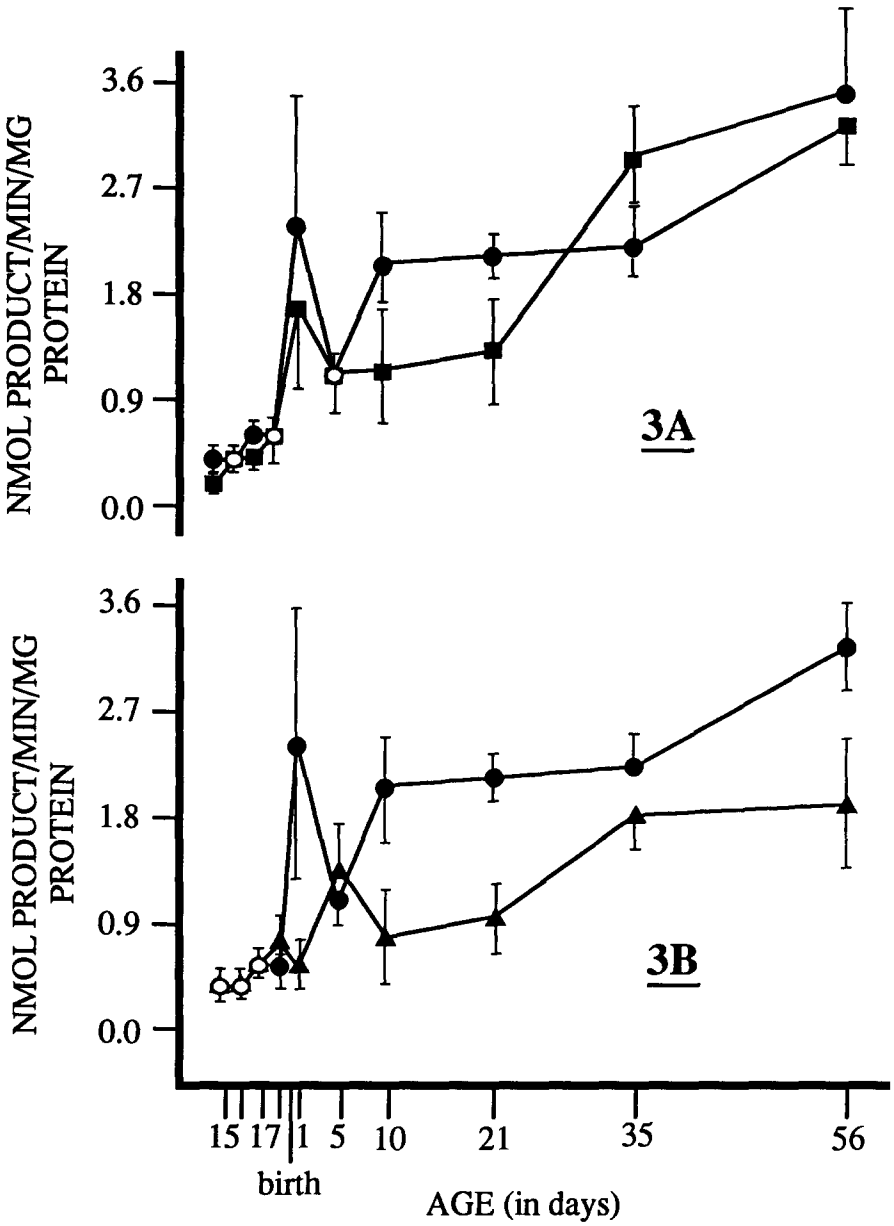

Figure 3. Prenatal and postnatal development of MAO in liver of Sprague-Dawley rats. Points are average ± s.e.m. for all litters in each age and treatment group. Figure 3A = comparison of control group (●) and DES group (■). Figure 3B = comparison of control group (●) and 4-CB group (▲) (significance between curves: $p < 0.005$).

differences in the overall developmental pattern of MAO have been reported in previous studies (Bennett and Giarman 1965; Kuzuya and Nagatsu 1969; Vaccari et al. 1972; Mantle et al. 1976; Nair et al 1976; Gripois and Fernandez 1977), however, direct comparisons are difficult because of differences in the substrate utilized and in the strain of animals examined.

Our results show substantial increases of short duration ($\leq$ 5 days) in specific activity of MAO at birth in both brain and liver. The occurrence of the activity spike near birth corresponds to large increases in thyroxine secretion in mammals two to three days before birth (Fischer 1975). Increases in MAO activity in brain caused by exogenous thyroxine have been documented by Gripois and Fernandez (1977). Consequently, the increase in MAO activity near birth may occur in response to increased thyroxine secretion. The syntheses of flavin, an MAO cofactor, is also affected by thyroxine (Rivlin et al 1976), although the data suggest that an increase in synthesis of the enzyme itself is most likely responsible for the elevated levels at parturition.

The increase in MAO activity accompanying birth is delayed about 48 hours in brain and liver of 4-CB-dosed rats (Figures 2B and 3B). In addition, gestation is prolonged, and birth weights are 12% lower in rats of the same treatment group (Rands et al. 1982). It is possible, therefore, that the delayed onset simply reflects a non-specific retardation in the overall development of exposed fetuses. However, 4-CB treatment also results in a significant reduction in MAO level throughout postnatal life. It is possible that this is a direct response to 4-CB, as 50% of the compound may remain uncleared from the intestine by day 5 after birth (Lucier et al. 1977).

Alternatively, the PCB effect may be indirect. Bastomsky (1974) reported that 25 mg Arochlor 1254/kg/4 d, administered intraperitoneally causes a four to five-fold increase in biliary clearance of thyroxine due to increases in its glucuronidation by UDP-glucuronyl transferase. A similar study in rats (Allen-Rowlands et al. 1979), but involving polybrominated biphenyl (PBB), showed that thyroxine is significantly reduced by 3 mg PBB/kg/d, administered orally for 10 days, but testosterone is not affected. The delay and reduction in MAO activity in both tissues in 4-CB-dosed animals (Figure 3B) might therefore be due to a primary effect of the compound on thyroxine secretion.

Administration of estrogen, including DES, to adult male rats results in inhibition of testosterone synthesis *in vitro* and *in vivo* (Sholiton et al. 1975; Bartke et al. 1977). However, the effects of DES on testosterone during development have not been carefully examined. Although the compound has a half-life of 24 hours in treated dams, significant quantities localize in the fetal reproductive tract (Shah and McLachlan 1976). The effect of DES in reducing testosterone in 35-day-old males, then, may be the result of a transient impairment of sexual imprinting of the Cells of Leydig in early neonates. An effect was not observed after completion of sexual maturation (day 70 of postnatal development).

In contrast to the temporary depression of testosterone levels produced by DES, the steroid is reduced both before (70% lower) and after (40% lower) puberty in the male offspring of 4-CB dosed mothers. The mechanism of this effect may involve the induction of detoxication enzymes which function in steroid catabolism. For example, Orrenius et al. (1965) reported that increases in cytochrome P-450 hydroxylase, which is induced for one month by a single injection (ip) of 80 ppm of PCB (Parkki et al. 1977), caused increased testosterone hydroxylation. The increase in hydroxylated testosterone could lead to an increase in excretion of the hormone by glucuronidation. The effect would be persistent because of the long half-life of the induced state of the P-450 hydroxylase. Finally, the disparate response

to DES and 4-CB by the enzymes and hormones studied suggest that the toxic effects of these compounds are mediated by quite different biochemical mechanisms.

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