Biochemical Pharmacology, Vol. 24, pp. 335–340. Pergamon Press. 1975. Printed in Great Britain.

# 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN-INDUCED CHANGES IN THE HYDROXYLATION OF BIPHENYL BY RAT LIVER MICROSOMES

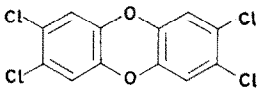
GARY E. R. HOOK, TERRY C. ORTON, JOHN A. MOORE and GEORGE W. LUCIER

National Institute of Environmental Health Sciences, Research Triangle Park, N.C. 27709, U.S.A.

(*Received* 18 *March* 1974; *accepted* 31 *May* 1974)

**Abstract**—Biphenyl 2- and 4-hydroxylase activities and cytochrome P-450 concentrations in microsomes were increased by oral doses of less than 1 μg TCDD/kg. Female rats were more sensitive than male rats to the inductive effects of TCDD, since highly significant increases in biphenyl-hydroxylating activities were observed at the dose level of 0·2 μg TCDD/kg in female but not in male rats. The inductive effect was very persistent: biphenyl 2- and 4-hydroxylases remained stimulated even after 73 days following a single oral dose of 25 μg TCDD/kg. The levels to which the hydroxylases were stimulated in female rats were the same as in male rats. Rats of all ages from 10 to 335 days responded to hepatic microsomal effects of TCDD to approximately the same degree. The enzyme inductive effect was diminished by the simultaneous administration of actinomycin D. The $K_m$ of biphenyl 2-hydroxylase (1·42 mM) was not altered significantly by TCDD treatment, but the $K_m$ of biphenyl 4-hydroxylase (0·62 mM) was increased to approximately the same value (1·6 mM) as that of the 2-hydroxylase. The $V_{max}$ of biphenyl 4-hydroxylase was increased 4·5-fold but that of biphenyl 2-hydroxylase was increased 16·5-fold. Rates of 2β- and 16α-hydroxylation of testosterone were suppressed by TCDD but rates of 7α- and 6β-hydroxylation were unaffected. It would appear that the hepatic microsomal mixed-function oxidases responsible for the hydroxylation of biphenyl and testosterone are different.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to be a teratogenic contaminant of certain commerical preparations of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) [1]. TCDD and a number



2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

of closely related compounds appear to be extremely toxic [1–3] and have been implicated in outbreaks of chick edema [2, 4], chloracne [5, 6] and porphyria cutanea tarda [7]. TCDD can also produce hepatic necrosis [6, 8, 9], fetal malformations [1, 10] and a number of other biological responses [9].

The lesions produced at the molecular level which underlie the observed toxicity of TCDD have never been elucidated, although recently the response of a number of biochemical parameters has been investigated [11–13]. Hepatic δ-aminolevulinic acid synthetase levels in the chick embryo are increased after the administration of TCDD [14] and so too are a number of mammalian hepatic microsomal mixed-function oxidases [15–17] as well as UDP glucuronyltransferase [18].

Biphenyl is hydroxylated only in the 4-position by the adult rat, although 2-hydroxylation also occurs in immature rats [19] and to a very small extent, if at all, in adult rats. The effect of TCDD on the microsomal hydroxylation of biphenyl is of considerable interest not only from the aspect of arylhydroxylase response to environmental agents, but also from the aspect of the changes which must occur at or near the active site of the cytochrome P-450 molecule to allow biphenyl to be hydroxylated at the 2-position.

## MATERIALS AND METHODS

*Treatment of rats.* TCDD (Lot No. 851-144-II; purity > 99 per cent) was supplied as a gift by the Dow Chemical Co. (Michigan).

Random bred albino rats of the Charles River CD stock were maintained on a rodent diet (Wayne Sterilizable Lab-Blox, Allied Mills, Inc., Chicago, Ill.) with free access to water. Unless otherwise indicated, TCDD was administered via stomach tube as a single dose in 0·5 ml acetone–corn oil (1:6·3). Animals were housed individually, thus eliminating the chances of cross-contamination of TCDD from animal to animal. For those experiments involving immature rats, pregnant female rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. The animals were mated so as to deliver on the same day. In the estimation of age, the day of delivery was taken as day 0. All litters were reduced to six pups and, at the indicated times, two male rats in each litter were treated with TCDD. Two males in each control litter

were also treated with acetone–corn oil. In actual experiments, only one animal was used from each litter, although two were treated in case of fatalities. Litters containing TCDD-treated animals did not contain acetone–corn oil-treated controls to prevent the possible spread of TCDD.

*Preparation of microsomes.* Animals were killed by cervical dislocation, livers were removed and immediately homogenized in 2 vol. of 1·15% KCl buffered with 0·02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7·5, at 5°, using a Teflon-glass homogenizer (four passes of the Teflon pestle: 0·006 to 0·009 in. clearance between Teflon pestle and smooth glass grinding chamber; 2500 rev/min). The homogenate was diluted to 4 vol. and then centrifuged at 10,000 $g_{av}$ for 20 min. The pellet was discarded and the supernatant was centrifuged at 192,000 $g_{av}$ for 45 min. The microsomal pellet was resuspended (free of most of the glycogen) in KCl–HEPES and then resedimented. The washed microsomes were finally suspended in KCl–HEPES to a concentration of 6·0 mg microsomal protein/ml. Protein concentrations were determined using the method of Lowry *et al.* [20].

*Biphenyl hydroxylation.* The hydroxylation of biphenyl in both the 2- and the 4-positions was measured according to the method of Creaven *et al.* [19] with one major difference. Biphenyl (Eastman Organic Chemicals, Rochester, N.Y.) was added to the incubation mixture in carboxymethyl-cellulose suspension [21] instead of Tween 80 solution. Incubation mixtures consisted of the following: 1·0 mg microsomal protein/ml; 0·17 M HEPES; 3·33 mM NADP; 10 mM glucose 6-phosphate; 1·33 U* glucose 6-phosphate dehydrogenase/ml; 10 mM biphenyl; 4·2 mg sodium carboxymethylcellulose/ml. The total volume of the incubation mixture was 1·5 ml and the pH was 7·55 at 37°. The NADPH-generating system was incubated at 37° for 15 min before adding it to the incubation mixtures. Reactions were started by the addition of the NADPH-generating system and were allowed to proceed for 15 min. Analysis of incubation mixtures for 2- and 4-hydroxybiphenyl was carried out as described by Creaven *et al.* [19].

*Testosterone hydroxylation.* The hydroxylation of testosterone in the 2β-, 6β-, 7α- and 16α-positions by rat liver microsomes was assayed by a modification of the radiochromatographic technique of Conney and Klutch [22]. Incubation mixtures consisted of microsomes (1 mg microsomal protein), 450 nmoles testosterone (0·5 μCi) (New England Nuclear, Boston, Mass.) added in 20 μl methanol, and 2 μmoles NADPH plus 0·1 M potassium phosphate buffer, pH 7·4, to a final volume of 2·0 ml. Reactions were started by the addition of NADPH. Incubation blanks (no NADPH) were carried through the same experimental procedure. Reactions were terminated after 9 min by the addition of 10 ml methylene chloride, shaken for 30 min and then centrifuged. An 8-ml aliquot of the organic phase was evaporated to dryness under nitrogen. The residue was dissolved in 60 μl methanol, and 25-μl aliquots were applied to 20 × 20 cm Silica gel GF plates (Analtech, Newark, Del.) activated at 110° for 10 min. The plates were developed in methylene chloride–acetone (90:50). The location of metabolites was determined by comparison with authentic standards. Radioactive metabolites were quantified by scraping the gel from the plates into scintillation vials containing 10 ml dioxane-based scintillator and counting in a scintillation counter.

*Cytochrome P-450 determination.* Cytochrome P-450 contents were determined by the CO-difference method of Omura and Sato [23] using a Beckman ACTA III recording spectrophotometer. Protein concentrations were reduced to 2·0 mg/ml for the cytochrome P-450 analyses and the suspending medium was 0·1 M HEPES buffer, pH 7·5.

*Actinomycin D experiments.* Actinomycin D was obtained from the Sigma Chemical Co. (St. Louis, Mo.). In those experiments involving actinomycin D, the animals were injected intraperitoneally with 1·0 mg actinomycin D/kg in 0·5 ml of 1·15% KCl.

## RESULTS

The hepatic microsomal hydroxylation of biphenyl in both the 2- and the 4-positions is highly sensitive to stimulation or induction by TCDD. This appeared to be especially true in the adult female rat, since highly significant increases ($P < 0·01$) in hydroxylase activities occurred at the TCDD dosage of 0·2 μg/kg (Fig. 1). The adult male rat appeared to be less responsive to the effects of TCDD than the female, since at 0·2 μg/kg changes in the activities of the hydroxylases were not significant in males. The levels (specific activities) to which the hydroxylases were stimulated by a dose of 5 μg TCDD/kg were similar for both male and female rats. The levels to which biphenyl 2- and 4-hydroxylases were stimulated in male rats by a single oral dose 3 days before sacrifice were not increased by multiple doses.† The ratio of the activities of biphenyl 4-hydroxylase and biphenyl 2-hydroxylase at the highest dose level (Fig. 1) was the same for both the male and female adult rats (4:2 = 2:1). Hepatic microsomal cytochrome P-450 also responded to the administration of TCDD (Fig. 1), although a strict proportionality between the cytochrome and either of the hydroxylases was not maintained in microsomes from either sex.

The inductive effect after a single oral dose of TCDD (25 μg/kg) was unusually persistent (Table 1). Of the two hydroxylases, biphenyl 4-hydroxylase was the quicker to respond and the more persistent. The stimulation of biphenyl 4-hydroxylase was at the maximum obtained 1 day after the single dose of TCDD and even 38 days after this dose showed no evidence of decreas-

* U = 1 unit = amount of glucose 6-phosphate dehydrogenase required to oxidize 1·0 μmole glucose 6-phosphate to 6-phosphogluconate/min at pH 7·4 at 25°, in the presence of NADP.

† Unpublished observations.

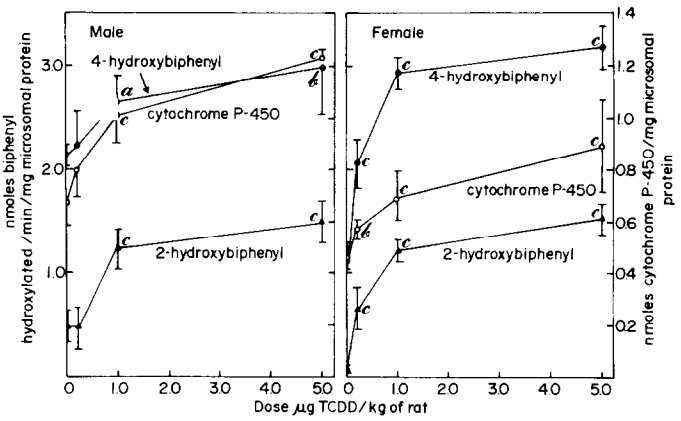



Fig. 1. Induction of cytochrome P-450 and biphenyl 2- and 4-hydroxylases in liver microsomes after treatment of male and female rats with TCDD. Four male rats (190–200 g) and four female rats (150–160 g) were treated at each dose level indicated. The rats received a single oral dose of TCDD in 0·5 ml corn oil–acetone (6·3:1). Control rats received corn oil–acetone only. The rats were sacrificed 3 days after dosing. The modification by Williams [24] of Dunnetts' test was used to test for treated–control differences at each dose level. Key: (a) $P < 0{\cdot}10$; (b) $P < 0{\cdot}05$; and (c) $P < 0{\cdot}01$.

Table 1. Persistence of induction after a single oral dose of TCDD*

| Days after dose | Biphenyl 4-hydroxylase† | | Biphenyl 2-hydroxylase‡ | |
|---|---|---|---|---|
| | Control | Treated | Control | Treated |
| 1 | 1·90 ± 0·19§ | 3·48 ± 0·11 | 0·10 ± 0·01 | 1·09 ± 0·10 |
| 3 | 1·67 ± 0·11 | 3·14 ± 0·16 | 0·22 ± 0·08 | 2·63 ± 0·09 |
| 10‖ | 1·47 ± 0·09 | 2·85 ± 0·35 | 0·12 ± 0·05 | 2·05 ± 0·37 |
| 38 | 1·97 ± 0·18 | 3·43 ± 0·28 | <0·05 | 0·83 ± 0·08 |
| 73 | 1·36 ± 0·25 | 2·24 ± 0·18 | 0·06 ± 0·02 | 0·35 ± 0·06 |

* Eight male rats weighing 190–200 g were used at the beginning of each time interval (day 0). Four of the rats received a single oral dose of 25 μg TCDD/kg in acetone–corn oil (1:6·3) (2·5 ml/kg). The remaining four rats were used as controls and received acetone–corn oil only. All treated–control differences were significant at $P < 0{\cdot}01$ level (two-sided Student's *t*-test).

† nmoles 4-hydroxybiphenyl formed/min/mg of microsomal protein.

‡ nmoles 2-hydroxybiphenyl formed/min/mg of microsomal protein.

§ Mean of values with samples from four individual rats ± S. D.

‖ The treated group contained only three animals.

Table 2. Induction of biphenyl 2- and 4-hydroxylases by TCDD in male rats of different ages*

| Age at time of sacrifice (days) | Biphenyl 4-hydroxylase† | | Biphenyl 2-hydroxylase‡ | |
|---|---|---|---|---|
| | Control | Treated | Control | Treated |
| 10 | 1·04 ± 0·13§ | 3·75 ± 0·26 | 0·03 ± 0·06 | 1·33 ± 0·15 |
| 20 | 1·37 ± 0·14 | 2·14 ± 0·48‖ | 0·24 ± 0·13 | 1·83 ± 0·50 |
| 38 | 1·75 ± 0·26 | 3·24 ± 0·57 | 0·26 ± 0·08 | 1·67 ± 0·25 |
| 80 | 1·33 ± 0·17 | 2·60 ± 0·28 | 0·16 ± 0·01 | 1·32 ± 0·16 |
| 335 | 1·45 ± 0·26 | 2·93 ± 0·41 | 0·22 ± 0·11 | 2·07 ± 0·35 |

* Eight male rats were used at each age. Four of the rats received a single oral dose of 25 μg/kg in acetone–corn oil (1:6·3) (2·5 ml/kg) and the remaining four acetone–corn oil only. Rats were sacrificed 3 days after dosing. All treated–control differences are significant at $P < 0{\cdot}01$ level unless indicated otherwise (two-sided Student's *t*-test).

† nmoles 4-hydroxybiphenyl formed/min/mg of microsomal protein.

‡ nmoles 2-hydroxybiphenyl formed/min/mg of microsomal protein.

§ Mean of values with samples from four individual rats ± S. D.

‖ $P < 0{\cdot}05$.

Table 3. Effect of actinomycin D on the induction of biphenyl 4-hydroxylase and cytochrome P-450 by TCDD*

| | Biphenyl 4-hydroxylase† | Cytochrome P-450‡ |
|---|---|---|
| Control | 2·86 ± 0·19§ | 1·01 ± 0·08 |
| TCDD | 4·68 ± 0·44‖ | 1·20 ± 0·04¶ |
| Actinomycin D | 3·19 ± 0·22** | 1·01 ± 0·20** |
| Actinomycin D + TCDD | 3·86 ± 0·46‖ | 0·94 ± 0·25** |

* Four male rats, each weighing between 190 and 200 g, were used in each group. TCDD (25 μg/kg) was given orally in a single dose in 0·5 ml acetone–corn oil (1:6·3). Actinomycin D (1 mg/kg) was injected intraperitoneally in 0·5 ml of 1·15% KCl. Control animals received 0·5 ml acetone–corn oil only. The group which received actinomycin D were also given 0·5 ml acetone–corn oil orally. Rats were sacrificed 18 hr later. Multiple comparison procedures employing an experiment-wide error rate were used to test for significant differences [27]. Only the results of treated–control comparisons are shown in the table.

† nmoles 4-hydroxybiphenyl formed/min/mg of microsomal protein.

‡ nmoles cytochrome P-450/mg of microsomal protein.

§ Mean of values with samples from four individual rats ± S. D.

‖ $P < 0·01$.

¶ $P < 0·05$.

** Not significant.

ing. Biphenyl 2-hydroxylase was slower to respond and 38 days after TCDD administration had decreased to less than one-third of maximum stimulation seen.

In spite of the fact that the level of activity of the hepatic 2-hydroxylation of biphenyl seems age dependent insofar as biphenyl 2-hydroxylase is often found only in immature animals [19], the age of the rat seemed to have little effect on the stimulation of the microsomal hydroxylase by TCDD (Table 2). The highest per cent increase in both 4- and 2-hydroxylation rates occurred in the 10-day-old rat, but this was a function of the low control values at this age. The levels (absolute specific activity) to which TCDD can increase biphenyl 4-hydroxylase and biphenyl 2-hydroxylase appear to be similar in male rats of all ages. Studies by Creaven and Parke [25] have shown biphenyl 2-hydroxylase to be inducible in immature rats, but stimulation of this activity in adult rats has not previously been reported to our knowledge. Such induction is of particular significance in light of the report by Basu *et al.* [26], that in male rats older than about 80 days, the level of hepatic microsomal biphenyl 2-hydroxylase activity is very low or absent. In some contrast to the results of Creaven and Parke [25] and Basu *et al.* [26] the 2-hydroxylation of biphenyl in our experiments occurred to a small degree

Table 4. Effect of TCDD on the apparent $K_m$ and $V_{max}$ of biphenyl 2- and 4-hydroxylases of rat liver microsomes*

| | $K_m$ (mM) | $V_{max}$ (nmoles product formed/min/mg protein) |
|---|---|---|
| Biphenyl 2-hydroxylase | | |
| Control | 1·42 ± 0·30† | 0·45 ± 0·12 |
| TCDD-treated | 1·35 ± 0·25‡ | 7·41 ± 0·53§ |
| Biphenyl 4-hydroxylase | | |
| Control | 0·62 ± 0·14 | 1·21 ± 0·39 |
| TCDD-treated | 1·60 ± 0·29§ | 5·47 ± 0·88§ |

* Four male rats (190–200 g) were dosed orally with TCDD (25 μg/kg) in 0·5 ml acetone–corn oil (1:6·3). Four control rats (190–200 g) were dosed orally with 0·5-ml vol. of acetone–corn oil only. The rats were sacrificed 4 days later. Microsomes were prepared from the livers of each individual rat. Incubations were carried out as described under Methods excepting that NADPH (3·3 mM in the incubation mixture) was used in place of the NADPH-generating system. The apparent $K_m$ and $V_{max}$ for microsomes from each rat liver were determined by a computerized least-squares technique from the Lineweaver–Burk plots. The regression line was weighted according to Cleland [28]. Statistical comparisons were made using multiple comparison procedures employing an experiment-wide error rate [27]. The comparisons shown in the table refer only to control–treated differences within either the 2-hydroxylase or 4-hydroxylase group. Other comparisons which should be noted are: (1) the $K_m$ of biphenyl 4-hydroxylase of TCDD-treated rats is not significantly different from the $K_m$ of biphenyl 2-hydroxylase, and (2) the $V_{max}$ of biphenyl 2- and 4-hydroxylases from TCDD-treated rats differs significantly at the $P < 0·05$ level.

† Mean of values with samples from four individual rats ± S. D.

‡ Not significant.

§ $P < 0·01$.

Table 5. Effect of TCDD on the hepatic microsomal hydroxylation of testosterone*

| | Testosterone hydroxylated (nmoles/min/mg microsomal protein) | | | |
|---|---|---|---|---|
| Position of hydroxylation | 2β | 6β | 7α | 16α |
| Control | 1·64 ± 0·22† | 2·80 ± 0·53 | 0·45 ± 0·08 | 2·05 ± 0·30 |
| TCDD-treated | 0·81 ± 0·14‡ | 2·73 ± 0·55§ | 0·46 ± 0·06§ | 0·93 ± 0·21‡ |

* Four male rats were dosed orally with 15 μg TCDD/kg in acetone–corn oil. Four control rats received acetone corn oil only. The rats weighed between 190 and 200 g at the time of dosing, and they were sacrificed 3 days later. Statistical comparisons were made using a two-sided Student's *t*-test.

† Mean of values with sample from four individual rats ± S. D.

‡ $P < 0{\cdot}01$.

§ Not significant.

in control rats of all ages studied. However, this difference between laboratories may be due to rat strain differences.

That the apparent increase in the hydroxylation of biphenyl upon treating rats with TCDD may be due, at least in part, to increased enzyme synthesis was indicated by the diminished stimulation of biphenyl 4-hydroxylase when actinomycin D was administered at the same time as TCDD (Table 3). Changes of cytochrome P-450 content proportional to those seen with biphenyl 4-hydroxylase were not obtained (Table 3).

Of considerable interest are the changes in the apparent $K_m$ and $V_{max}$ wrought by pretreatment of rats with TCDD (Table 4). TCDD did not change the apparent $K_m$ of biphenyl 2-hydroxylase, although the $V_{max}$ was increased 16·5-fold. The induced enzyme is probably the same as that found in the control animals excepting that its activity in the microsomal membrane has increased enormously. Both kinetic constants of biphenyl 4-hydroxylase, however, underwent considerable changes. Although $V_{max}$ of biphenyl 4-hydroxylase increased 4·5-fold, of special interest was the observed increase in the apparent $K_m$ to a value indistinguishable statistically from that of the apparent $K_m$ of the 2-hydroxylase (Table 4).

The effects of treatment of rats with TCDD on the hepatic microsomal hydroxylations of testosterone are shown in Table 5. Testosterone hydroxylation in the 2β- and 16α-positions appeared to be suppressed ($P < 0{\cdot}01$) by treatment of rats with TCDD; whereas the hydroxylations in the 6β- and 7α-positions were unaffected.

## DISCUSSION

TCDD appears to be one of the most potent inducers of the hepatic microsomal foreign-compound-metabolizing enzyme systems as yet reported—at least as far as biphenyl 2- and 4-hydroxylases are concerned. Not only are the biphenyl hydroxylases extremely sensitive to induction by TCDD, but the effects of this compound are very long lasting. Many polychlorinated compounds will induce certain hepatic mixed-function oxidases (see for example Refs. 29 and 30), and persistence of induction is typical of animal exposure to many of these compounds [30], but none has been shown to be as long lasting as TCDD.

In the untreated male rat, biphenyl appears to be hydroxylated by two different enzymes, the major one of which is responsible for hydroxylation in the 4-position and the other, present to a minor degree, is responsible for hydroxylation in the 2-position. Treatment of rats with TCDD appears to stimulate the synthesis of biphenyl 2-hydroxylase, and that this induced enzyme is the same as that present in small quantities in untreated rats is indicated by the fact that the apparent $K_m$ remains unchanged after TCDD treatment. Biphenyl 4-hydroxylase is also induced, but the apparent $K_m$ is increased considerably, interestingly to a value similar to that of biphenyl 2-hydroxylase. Such a situation could arise if the induced enzyme were responsible for both 2- and 4-hydroxylation of biphenyl.

Competitive inhibition of biphenyl 4-hydroxylase by TCDD could also result in an increased $K_m$. However, the maximum concentration of TCDD which could be present in an incubation mixture, assuming that the total oral dose of TCDD was recovered in the microsomes, could only produce a concentration of $3 \times 10^{-8}$ M. Allosteric inhibition is also possible, but again it must be pointed out that, even if all the TCDD given was located in the endoplasmic reticulum, there would be at least 30 times as much cytochrome P-450 present than TCDD.

If in the TCDD-treated rats a single enzyme is responsible for both 2- and 4-hydroxylation, then it would also appear that neither 2- nor 4-hydroxylation is favored by the enzyme, since $V_{max}$ is similar for both processes. This would suggest that the stereochemistry of the active site in the TCDD-induced enzyme favors neither "edge-on" nor "end-on" orientations of the biphenyl molecule [31]. Thus, the TCDD-induced enzyme may differ from biphenyl 4-hydroxylase as found in the untreated rats by having a more open type of structure, perhaps brought about by membrane alterations.

As an effector of hepatic mixed-function oxidases, TCDD bears a resemblance to 3-methylcholanthrene (3-MC) [16], since the CO complex of the dithionite-

reduced cytochrome has an absorption maximum at 448 nm in the CO difference spectrum. Although both TCDD and 3-MC give rise to cytochrome P-448, the inductive effects of these compounds on microsomal mixed-function oxidases are not identical. For example, 3-MC is an inducer of mammalian hepatic δ-aminolevulinic acid synthetase [32], but TCDD is not. TCDD is an inducer of biphenyl 4-hydroxylase, but 3-MC is not [25]. The effects of TCDD on the hydroxylation of testosterone also differ from those of 3-MC. TCDD affects neither 6β- nor 7α-hydroxylation of testosterone and suppresses both 2β- and 16α-hydroxylation. In these effects on hepatic testosterone metabolism, TCDD resembles Chlorthion [33]. 3-MC stimulates 7α-hydroxylation, suppresses 16α-hydroxylation and has no effect on 6β-hydroxylation of testosterone. These differences suggest that if stereospecific alterations in the active site and/or membrane environment of cytochrome P-450 are responsible for changes in the position of hydroxylation, then the alterations in active site, etc., produced by TCDD differ from those produced by 3-MC, and different enzymes might be responsible for the hydroxylation of biphenyl and testosterone.

TCDD is a very slow-acting toxin. Regardless of dose, animals die weeks after a single administration of TCDD. The effect of TCDD on the hydroxylation of biphenyl is extremely persistent. Such long-lasting effects on a metabolic process or system such as a hepatic mixed-function oxidase could underlie the slow nature of the toxic action of TCDD. TCDD can simultaneously both activate and suppress certain membrane-associated foreign-compound- and steroid-hormone-metabolizing enzyme systems. Either process, over a long period of time, could result in the accumulation of an ultimately toxic material leading to delayed death.

*Acknowledgements*—We thank Robert E. Easterling, Linda B. Gilmore, Otelia S. McDaniel, W. Douglas Taylor and Patricia A. Singletary for excellent technical assistance, Martha Harris for treating some of the animals with TCDD, and Dr. Joseph K. Haseman of the Biometrics Branch (NIEHS) for the statistical analyses. We are also grateful to Dr. James R. Fouts for advice and discussion throughout the course of the investigation.

## REFERENCES

1. G. L. Sparschu, F. L. Dunn and V. K. Rowe, *Fd Cosmet. Toxic.* **9**, 405 (1971).
2. G. R. Higginbotham, A. Huang, D. Firestone, J. Verrett, J. Ress and A. D. Campbell, *Nature, Lond.* **220**, 702 (1968).
3. B. A. Schwetz, J. M. Norris, G. L. Sparschu, V. K. Rowe, P. J. Gehring, J. L. Emerson and C. G. Gerbig, *Envir. Hlth Perspect.* **5**, 87 (1973).
4. J. S. Cantrell, N. C. Webb and A. J. Mabis, *Chem. Eng. News* **45** (No. 5), 10 (1967).
5. J. Kimmig and K. H. Schulz, *Dermatologica* **115**, 540 (1957).
6. K. H. Schulz, *Arch. Klin. Exp. Derm.* **206**, 589 (1957).
7. A. Poland, D. Smith, G. Metter and P. Possick, *Archs envir. Hlth* **22**, 316 (1971).
8. B. N. Gupta, J. G. Vos, J. A. Moore, J. G. Zinkl and B. C. Bullock, *Environ. Hlth Perspect.* **5**, 125 (1973).
9. D. H. Norback and J. R. Allen, *Environ. Hlth Perspect.* **5**, 233 (1973).
10. K. D. Courtney and J. A. Moore, *Toxic. appl. Pharmac.* **20**, 396 (1971).
11. N. P. Buu-Hoi, D.-P. Hien, G. Saint-Rut and J. Servoin-Sidoine, *C.r. hebd. Séanc. Acad. Sci. [D], Paris* **272**, 1447 (1971).
12. N. P. Buu-Hoi, P.-H. Chanh, G. Sesqué, M. C. Azum-Gelade and G. Saint-Rut, *Naturwissenschaften* **59**, 173 (1972).
13. J. B. Greig, *Biochem. Pharmac.* **21**, 3196 (1972).
14. A. Poland and E. Glover, *Science, N.Y.* **179**, 476 (1973).
15. G. W. Lucier, O. S. McDaniel, G. E. R. Hook, B. A. Fowler, B. R. Sonawane and E. Faeder, *Environ. Hlth Perspect.* **5**, 199 (1973).
16. J. B. Greig and F. DeMatteis, *Environ. Hlth Perspect.* **5**, 211 (1973).
17. A. Poland and E. Glover, *Environ. Hlth Perspect.* **5**, 245 (1973).
18. G. W. Lucier, O. S. McDaniel and G. E. R. Hook, *Biochem. Pharmac.* **24**, 325 (1975).
19. P. J. Creaven, D. V. Parke and R. T. Williams, *Biochem. J.* **96**, 879 (1965).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. G. E. R. Hook, J. R. Bend and J. R. Fouts, *Chem. Biol. Interact.* **7**, 205 (1973).
22. A. H. Conney and A. Klutch, *J. biol. Chem.* **238**, 1611 (1963).
23. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
24. D. A. Williams, *Biometrics* **28**, 519 (1972).
25. P. J. Creaven and D. V. Parke, *Biochem. Pharmac.* **15**, 7 (1966).
26. T. K. Basu, J. W. T. Dickerson and D. V. W. Parke, *Biochem. J.* **124**, 19 (1971).
27. R. G. Miller, in *Simultaneous Statistical Inference*, pp. 90–3. McGraw–Hill, New York (1966).
28. W. W. Cleland, *Nature, Lond.* **198**, 463 (1963).
29. L. G. Hart and J. R. Fouts, *Proc. Soc. exp. Biol. Med.* **114**, 388 (1963).
30. L. G. Hart, R. W. Shultice and J. R. Fouts, *Toxic. appl. Pharmac.* **5**, 371 (1963).
31. H. Jaffe, K. Fujii, H. Guerin, M. Sengupta and S. S. Epstein, *Biochem. Pharmac.* **18**, 1045 (1969).
32. J. Baron and T. R. Tephly, *Biochem. biophys. Res. Commun.* **36**, 526 (1969).
33. R. M. Welch, W. Levin and A. H. Conney, *J. Pharmac. exp. Ther.* **155**, 167 (1967).