transferase might be of value in studying the relationship between the oxidative and conjugative reactions of the hepatic microsomes as related to the drug metabolism. Relationships between this finding and porphyria caused by long term exposure to HCB, if any, need to be investigated.

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Retention and Excretion of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin by Rats

George F. Fries* and George S. Marrow

Rats were fed 7 or 20 ppb of 2,3,7,8-[^{14}C]tetrachlorodibenzo-*p*-dioxin (TCDD) in their diets for 42 days. Dose-related effects on feed consumption and growth were more severe in males than in females. Both levels of TCDD significantly increased liver weights, but 7 ppb caused the greater increase. Total retention of TCDD was closely related to total intake at any given time period. Males and females did not differ significantly in total retention, but 85% of the TCDD was in the liver of males, whereas only 70% was in the liver of females. Total retention was 5.5, 7.5, and 10.0 times daily intake at 14, 28, and 42 days, respectively. Kinetic analysis indicated that at steady state, total retention would be approximately 10.5 times the average daily intake. When feeding stopped, TCDD residues were eliminated from the body with half-lives of 12 and 15 days for males and females, respectively.

Concern about the potential health hazards associated with the chlorinated dibenzo-*p*-dioxins has been expressed in recent years. Dioxins may arise as by-products in the manufacture of certain chlorinated phenols. Interest is mainly focused on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which appeared as a trace contaminant in some samples of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). TCDD has been associated with chloracne in humans and numerous pathological effects in laboratory animals (Environmental Health Perspectives, 1973). In addition, its single-dose oral LD_{50} is quite low for many species (Schwetz *et al.*, 1973). Because of its high potential for adverse effects, it is important to obtain information on the retention and elimination of TCDD in animals. Obtaining this information has been hindered because of the high toxicity of TCDD and the lack of an available analytical method that could be routinely used when animals are fed sublethal quantities.

Piper *et al.* (1973) studied the excretion and tissue distribution of [^{14}C]TCDD in the male rat after oral administration of a single dose approximately twice the LD_{50}. Among the tissues examined the liver had the highest concentration and contained about 50% of the ^{14}C activity during the first week after dosing. The concentration in liver was somewhat greater than in fat and both were about 10 times higher than any other tissue. Over 50% of the dose was eliminated *via* the feces in 21 days, with much smaller amounts eliminated *via* urine and expired air. Vinopal and Casida (1973) studid the fate of TCDD in mice injected with a single LD_{50} dose. As in rats, liver was the major site of concentration and no water-soluble products were detected. Feces was the major route of elimination, probably *via* the bile.

Single-dose studies indicated the major sites of retention and routes of elimination. However, they do not necessarily provide a good basis for evaluating steady-state burdens or rates of elimination resulting from continuous low-level exposure. In this study, we determined the rates

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Table I. Comparison of the Total Intake of TCDD Fed at Two Levels with the Single-Dose LD_{50}

Days	Male		Female	
	7 ppb, μg/kg	20 ppb, μg/kg	7 ppb, μg/kg	20 ppb, μg/kg
	LD_{50}[a]			
	22.0		45.0	
	Intake			
0–14	7.7	20.5	6.9	18.1
0–28	15.0	41.2	14.3	36.1
0–42	21.8	61.1	21.6	53.2

[a] LD_{50} from Schwetz *et al.* (1973).

of TCDD uptake in the liver and the whole body of rats fed TCDD continuously in their diets. We also determined elimination rates of TCDD when the animals were changed to an uncontaminated diet.

MATERIALS AND METHODS

Sixty rats (Sprague-Dawley strain) consisting of equal numbers of male and female were used. The rats were 80–90 days old and weighed 190–210 g at the start of the study. The rats, individually housed in metal metabolism cages, were randomly assigned to one of three groups consisting of ten male and ten female rats. The control group was fed a standard unpelletted laboratory diet (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.). The second and third groups were fed the same diet except that it contained 7 and 20 ppb of TCDD, respectively. The TCDD was uniformly labeled with ^{14}C (sp act. 2.8 μCi/mg) (obtained from The Dow Chemical Co., Midland, Mich.).

The spiked diets, in amounts sufficient for the entire study, were prepared by dissolving the required amount of TCDD in approximately 100 ml of benzene. The benzene solution was pipeted on 0.5 kg of the control diet spread in a pan. After the benzene evaporated the feed was thoroughly mixed. The premix was then added to 9.5 kg of the unpelleted laboratory diet and mixed in an enclosed Hobart Model VOM 40 mixer. Four samples of each diet were analyzed by combustion and the recovery of ^{14}C activity was 96.3 ± 5.7% of the theoretical amount added. These results suggest that the preparation and mixing methods were adequate to produce a uniform finished diet.

The experiment lasted 70 days. The TCDD diets were fed for 42 days, after which all rats received the control diet. Feed was offered *ad libitum* and consumption recorded. Total feces and urine were collected. Rats were weighed at 7-day intervals.

Two rats of each sex were randomly selected from each group at 14-day intervals and sacrificed. The rats were anesthetized with ether and the liver removed, weighed, and frozen for future analysis. The remainder of the carcass was homogenized with water and ice (Fries *et al.*, 1969).

Appropriate samples of the whole-rat and liver homogenates were digested by refluxing with a solution containing 25 g of KOH in 100 ml of ethanol. After the tissue was digested, the samples were diluted with water and extracted with petroleum ether. The petroleum ether extracts were concentrated and ^{14}C activity was determined by liquid scintillation counting. Recovery of [^{14}C]TCDD added to control samples was 90.2 ± 2.7%. It was assumed that all of the ^{14}C activity was unmetabolized TCDD (Vinopal and Casida, 1973).

An attempt was made to determine the ^{14}C activity in feces and urine by combustion. Because of the low specific activity of the TCDD used, however, activities in the feces and urine were generally too low to provide reliable counting statistics. Therefore, it was not possible to provide the balance data as originally intended.

The significance of differences among the means of the various parameters was tested at each observation period by analyses of variance (Snedecor, 1956). Mean separation, when required, was achieved by Turkey's test (Snedecor, 1956).

RESULTS AND DISCUSSION

Physiological Observations. Relatively high intakes of TCDD were required to provide enough ^{14}C activity for reliable counting statistics. The total TCDD intakes by the various groups are compared with single-dose LD_{50}'s determined by Schwetz *et al.* (1973) in Table I. In spite of total intakes three times the LD_{50} by males on the 20-ppb diet, all of the rats survived until sacrificed. This result is not too surprising, since the effects of a single dose of a toxic material are usually more severe than the effects of the same dose given over a longer time. This experiment was relatively short, and deaths might have occurred in a longer experiment.

Both male and female rats consumed significantly less feed when TCDD was fed at the rate of 20 ppb (Table II). Males and females responded differently to 7 ppb of TCDD, consistent with the sex difference in LD_{50}. Females did not reduce their feed consumption, but males ate significantly less through the first 14 days. After 14 days, consumption of the diet containing 7 ppb of TCDD was not significantly different from consumption of the control diet. Harris *et al.* (1973) found reduced feed intake with single doses of TCDD, but the differences were not significant at doses lower than 100 μg/kg. This dose is twice the maximum total dose used in our study.

After the feeding of TCDD was stopped at 42 days, there was evidence that the treated rats began to recover. At 43–56 days, the intake by the treated groups (except for the females fed 20 ppb) was not significantly different from the intake by the control groups. Since the growth of the treated groups was reduced, their feed consumption per unit of body weight was actually higher than that of

Table II. Effect of TCDD on Feed Consumption of Rats

Period, days	Rats, no.	Male			Female			Std dev
		0, g	7, g	20, g	0, g	7, g	20, g	
0–14	10	306[a]	276[b]	245[c]	198[d]	198[d]	174[e]	16
15–28	8	341[a]	314[a]	268[b]	227[c]	217[c]	178[d]	21
29–42	6	332[a]	318[ab]	287[b]	218[c]	222[c]	161[d]	22
43–56	4	344[a]	326[a]	308[a]	228[b]	239[b]	160[c]	24
57–70	2	306[a]	301[a]	297[a]	205[b]	216[b]	239[b]	22

[a-e] Means with a common superscript on any horizontal line are not significantly different ($P < 0.05$). TCDD was not fed after 42 days.

Table III. Effect of TCDD on the Average Weight Gain of Rats

Period, days	Rats, no.	Male 0, g	Male 7, g	Male 20, g	Female 0, g	Female 7, g	Female 20, g	All 0, g	All 7, g	All 20, g	Std dev
0–14	10	83.6[a]	67.6[b]	52.6[c]	19.5[de]	23.7[d]	11.6[e]	51.6	45.7	32.1	9.7
15–28	8	44.0	36.5	17.8	12.8	5.5	−2.5	28.4[a]	21.0[a]	7.7[b]	13.1
29–42	6	27.8	20.7	16.3	4.2	1.2	−7.8	16.0[a]	11.0[ab]	4.5[b]	8.1
43–56	4	31.5[ab]	28.8[ab]	40.8[a]	8.5[cd]	16.8[bc]	0.3[d]	20.0	22.8	20.6	6.7
57–70	2	15.5	22.5	29.0	12.0	6.0	50.0	13.8[a]	14.3[a]	39.5[b]	11.1

[a-e] Means on the same horizontal line with a common superscript are not significantly different ($P < 0.05$). The sex difference was significant in all periods except 57–70 days. The range test was not applied to the treatment means within sexes in those periods in which the sex × treatment interaction was not significant. TCDD was not fed after 42 days.

Table IV. Effect of TCDD on the Liver Weight of Rats

Days	Liver wt, % of body wt: 0, ppb	7, ppb	20, ppb	Std dev
14	4.74[a]	5.98[b]	5.75[b]	0.30
28	4.51	5.42	4.88	0.62
42	4.08[a]	5.98[c]	5.07[b]	0.41
56	3.66[a]	4.23[ab]	4.63[b]	0.45
70	3.87[a]	4.23[ab]	5.45[b]	0.60

[a-c] Means with a common superscript on a horizontal line are not significantly different ($P < 0.05$). Sex differences and sex × treatment interactions were not significant. Each value is a mean of two male and two female rats. TCDD was not fed after 42 days.

Table V. TCDD Concentration in Whole Body, Fat, and Liver of Rats[a]

Sex	Level, ppm	Days 14	28	42	56	72
		Whole Body Less Liver, ppb				
Male	7	0.04	0.04	0.10	0.04	0.02
	20	0.08	0.18	0.16	0.09	0.07
Female	7	0.07	0.09	0.22	0.12	0.14
	20	0.13	0.38	0.33	0.38	0.05
		Fat, ppb				
Male	7	0.69	1.07	1.52	0.33	0.29
	20	1.34	5.56	3.30	0.78	0.70
Female	7	0.99	0.91	1.99	1.97	1.06
	20	1.71	6.30	6.20	4.90	0.40
		Liver, ppb				
Male	7	3.7	4.4	5.8	3.0	1.0
	20	10.9	15.8	15.9	5.8	3.3
Female	7	2.8	4.9	6.1	3.3	2.3
	20	9.7	15.0	15.8	9.1	1.7

[a] TCDD concentration was based on ^{14}C activity assuming no metabolism. Each value was an average of two rats and TCDD was not fed after 42 days.

the controls. However, it was difficult to draw definite conclusions from the small number of rats remaining.

The effect of TCDD on weight gains (Table III) paralleled the effect on feed consumption both during and after treatment. This close agreement suggests that the reductions in growth were directly caused by the reductions in feed consumption. Harris *et al.* (1973) found that daily doses of 1 μg/kg of TCDD for 31 days significantly reduced the weight gain of female rats, and that recovery occurred when TCDD feeding stopped. The average intake by our 20-ppb female was 1.3 μg/kg (Table I) and the results of the two studies are consistent.

The increase in relative liver weight (Table IV) was the most sensitive parameter measured. The liver weights are expressed as a per cent of body weight because of the differences in body weight between sexes and among treatment groups. When expressed this way, sex differences and sex × treatment interactions were not significant. Therefore, the sexes have been combined in Table IV.

The increases in liver weights were not directly related to TCDD intake. Response was greater in the 7-ppb group than in the 20-ppb group while TCDD was fed. However, after TCDD feeding stopped, the 7-ppb group rapidly recovered to more normal liver weights and was not significantly different from the controls. The 20-ppb group continued to exhibit greater liver weights throughout the study.

Increases in liver weights have been noted in short-term, single-dose studies (Harris *et al.*, 1973), and are a common response to numerous chlorinated compounds. Increases in the liver weight are often associated with changes in liver structure and enzyme activity (Gupta *et al.*, 1973; Lucier *et al.*, 1973). The greater increase in liver weight with 7 ppb than 20 ppb in this study cannot be readily explained. Sections of the liver for histopathological examination were not taken.

Residues. TCDD was determined in homogenates of liver and the remaining whole body. Residues were also determined in fat extracted from lyophilized whole-body homogenates. The latter values should be roughly comparable to the TCDD levels in the fat of adipose tissues. This assumes that TCDD is stored in fat and is not concentrated in tissues other than liver (Piper *et al.*, 1973).

As shown in Table V, females had TCDD concentrations 2 to 3 times greater than males in the whole body (less liver). However, females contained more fat than did males, and the differences between sexes were largely eliminated when TCDD concentration was expressed on a fat basis. The results for whole body and fat are somewhat erratic, largely because of the low concentration of activity in the whole body. Thus, small absolute differences among animals and counting errors were large on a relative basis.

Concentrations of TCDD were highest in the liver, and were directly proportional to dietary concentrations. The small difference between the sexes at either level of intake was fairly remarkable because intake per unit body weight was different, the amount stored in body fat was different, and the liver sizes were considerably different. All of these factors could affect liver concentration, and the close agreement is probably fortuitous.

The concentration of TCDD in a given tissue was often quite variable due to differences in intake, liver size, and body composition. However, the total amount of TCDD retained in the body (including liver) at a given time was closely related to the total intake of TCDD. This relationship is illustrated for the rats sacrificed at 14 days (Figure

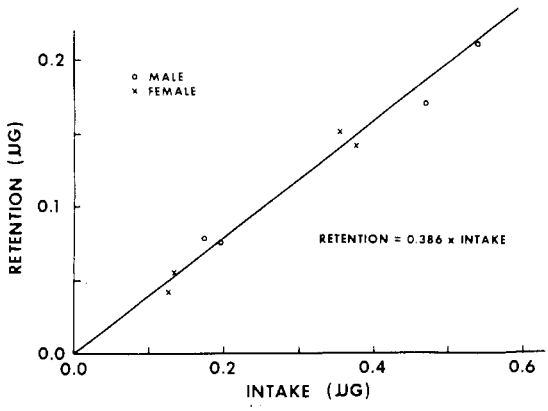

Figure 1. Total TCDD retained in rats as a function of total TCDD intake. The rats were sacrificed after feeding TCDD for 14 days. TCDD retention was based on ^{14}C activity assuming no metabolism.

Table VI. Retention of TCDD in Liver and Remaining Body Normalized to a Common TCDD Intake[a]

	Retention, multiple of daily intake[b]					
	Liver		Body		Total	
Days	Male	Female	Male	Female	Male	Female
14	4.84	4.22	0.73	1.20	5.57	5.42
28	6.38	5.56	1.12	1.83	7.50	7.39
42	8.36	6.39	1.81	3.55	10.17	9.94
56	2.93	2.91	0.84	2.77	3.77	5.68
70	1.33	1.64	0.63	1.72	1.96	3.36

[a] Each value is an average of two rats fed 7 ppb and two rats fed 20 ppb. TCDD retention was based on ^{14}C activity assuming no metabolism. TCDD was not fed after 42 days. [b] (Multiple of daily intake) = (total residue)/(total intake) (days fed).

1). There was more variation for periods longer than 14 days, because of the accumulated errors of measuring intake and because the possible small differences between rats in excretion would be more important. However, the results for longer periods were consistent with the general conclusion that total retention was proportional to total intake.

The total retention of TCDD in the liver and remaining body at the various time periods is summarized in Table VI. The values are expressed as a multiple of the average daily intake. This normalization is justified because of the close relationship between intake and retention (Figure 1) and is useful for making estimates of elimination rates and steady-state burdens. There were no significant differences between the dose levels, and the data were combined for ease of presentation.

The two sexes retained approximately equal amounts of total residue while TCDD was fed. However, the distribution of residue between the liver and remaining body differed significantly between males and females. About 85% of the total residue was in the liver of males, whereas only 70% of the residue was in the liver of females. When feeding of TCDD stopped, these percentages became smaller because the TCDD was removed from liver considerably faster than from the body.

Half-lives of TCDD residues in liver, remaining body, and in total body were estimated by the least-squares method using the linear form of the first-order equation (Table VII). The half-lives for residues in the body (less liver) cannot be considered reliable because of the large standard errors. The values for the total body are closely related to values for liver, reflecting the predominance of liver as a storage site of TCDD.

Table VII. Elimination of TCDD Residues from the Rat

Tissue	$-k$,[a] day^{-1}	SE[b]	Half-life, days
	Male		
Liver	0.065	±0.016	11
Remaining body	0.033	±0.028	21
Total	0.058	±0.016	12
	Female		
Liver	0.052	±0.027	13
Remaining body	0.040	±0.061	17
Total	0.046	±0.041	15

[a] From the linear form of the first-order equation: $\ln A = \ln A_0 - kt$, where A = amount of TCDD in tissue, A_0 = amount at t_0, t = time on clean feed, and k = constant. [b] Standard error.

Table VIII. Steady-State Retention of TCDD Estimated from Retention at the Various Sacrifice Dates

Days	Fraction of steady state[a]	Steady-state retention[b]
	Male	
14	0.56	10.0
28	0.80	9.4
42	0.91	11.2
	Female	
14	0.48	11.4
28	0.72	10.2
42	0.86	11.6

[a] A/A_s, calculated from eq 1 using k values from Table VII. [b] A_s as a multiple of daily intake, calculated by dividing the values of Table VI by A/A_s.

The 12-day half-life of TCDD in males observed in our study was considerably shorter than the 17.4 ± 5.6 days found by Piper *et al.* (1973) with single-dose studies. However, the dose used in their study was high (approximately twice the LD_{50}), the rats lost weight, and their physical condition deteriorated. These factors could affect elimination rate and, possibly, there are no discrepancies between the studies.

Steady-State Burdens. The results of Piper *et al.* (1973) and this study are consistent with the assumption that TCDD retention and elimination are adequately described by a single compartment with first-order rates. In this case, the kinetics become quite simple and the steady-state burdens of TCDD that will occur with continuous intake can be estimated. The retention of TCDD would be described by the equation:

$$A = A_s(1 - e^{-kt}) \quad (1)$$

where A is the retention at any time, A_s is the retention at steady state, e is the base of the natural logarithms, k is the first-order rate constant numerically equal to the constants presented in Table VII, and t is time in days.

At any time, one can estimate the retention as a fraction of the steady-state retention (A/A_s) by dividing both sides of eq 1 by A_s and evaluating the expression within parentheses. These values for the three sacrifice times are presented in Table VIII. The steady-state burden, in terms of daily intake, can be estimated by dividing these fractional values into the observed values for total body burden at various times (Table VI). The estimated steady-state burdens are presented in Table VIII.

Both males and females provide an estimate of the steady-state burden 10 to 11 times the daily intake at all periods of observation. Gehring (1974) has used the results of Piper *et al.* (1973) and a similar model to estimate a steady-state burden of 17.5 times the daily intake. The

difference can be attributed to the differences in half-lives observed. The consistency of these values at the various times in our study supports the concept that the kinetics of TCDD retention and elimination are adequately described by a single-compartment model.

A second method of calculating steady-state retention provides an estimate of TCDD absorption from the GI tract when compared to the values in Table VIII. At steady state, TCDD intake (eq 1) will equal TCDD excretion (kA_s). The total body burden is described by eq 2.

$$A_s = I/k \quad (2)$$

When the k values in Table VII were used, the A_s values for males and females were 17.2 and 21.7 times intake, respectively. These values are considerably higher than the values shown in Table VIII and imply that only 50–60% of the TCDD was absorbed. The results of Piper *et al.* (1973) suggest that 70% was absorbed in their study when TCDD was administered as a single dose in oil. It is not unreasonable to expect that TCDD incorporated in the feed is absorbed less efficiently than TCDD dissolved in oil.

These estimates implicitly assume that a number of parameters are constant, *i.e.*, concentration of TCDD in the diet, level of feed intake, body size, and body composition. In the "real world" none of these can be expected to be constant and a true steady state would never be reached. In addition, our values for TCDD were based on ^{14}C activity and assume that there was no metabolism. This is a valid assumption at this time but future studies may demonstrate that some of the activity was actually a metabolite.

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Metabolic Fate of Clopidol after Repeated Oral Administration to Rabbits

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After oral administration of single (16 mg/kg) or multiple doses (16 mg/kg per day) of [^{14}C]-3,5-dichloro-2,6-dimethyl-2,6-[^{14}C]pyridin-4-ol (clopidol) to rabbits most of the material was rapidly absorbed and almost exclusively excreted in the urine. Following repeated daily oral doses, no accumulation of radioactivity occurred in the tissues and no radioactivity was detected in the expired air. During a period of withdrawal after five daily doses, radioactivity was below the limits of detection in the tissues or plasma of rabbits killed later than 32 hr after administration of the last dose. Three major radioactive components were detected in the rabbit urine, two of these were identified by tlc and mass spectrometry as unchanged clopidol and 3,5-dichloro-2-hydroxymethyl-6-methylpyridin-4-ol accounting for a mean of 47 and 32%, respectively, of the urinary radioactivity. The third metabolite, accounting for a mean of 20% of the urinary radioactivity, was probably the *O*-glucuronide of the hydroxylated metabolite.

Clopidol, 3,5-dichloro-2,6-dimethylpyridin-4-ol, is widely used for the control of coccidiosis in chickens (Stevenson, 1965; Stock *et al.*, 1967) and its chemotherapy has been reviewed (Ryley and Betts, 1973). Smith and Watson (1969) showed that [^{36}Cl]clopidol was rapidly absorbed and excreted by rats after oral administration.The half-life of radioactivity in most tissues was about 10 hr and almost equal amounts were excreted in the urine and feces. The radioactivity excreted in the feces may have been due to unabsorbed clopidol or may have represented compounds excreted in the bile, but this was not determined. The metabolism of [^{36}Cl]clopidol in chickens has also been investigated (Smith, 1969) when the residues in tissues were identified as unchanged clopidol. As part of the safety evaluation for the use of this compound in other species we have studied its metabolic fate in rabbits after single and repeated doses.

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MATERIALS AND METHODS

3,5-Dichloro-2,6-dimethyl-2,6-[^{14}C]pyridin-4-ol, ([^{14}C]-clopidol) of specific activity 0.943 mCi/mmol, unlabeled clopidol, and reference compounds 3,5-dichloro-2-hydroxymethyl-6-methylpyridin-4-ol and 3,5-dichloro-2-carboxy-6-methylpyridin-4-ol were supplied by Dow Chemical Co., Kings Lynn, U.K. Thin-layer chromatography showed that [^{14}C]clopidol was >99% radiochemically pure.

Thin-Layer Chromatography. Thin-layer chromatography (tlc) was carried out on prelayered Kieselgel F_{254} plates (E. Merck A.G., Darmstadt, Germany) of layer thickness 0.25 or 2 mm. The solvent systems used and R_f values of reference compounds are shown in Table I.

^{14}C-Labeled metabolites were detected by autoradiography using Kodak Kodirex X-ray film. Radioactive areas of the gel were removed and counted in a Triton X-100 scin-