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NATURE OF THE ENHANCEMENT OF HEPATIC URIDINE DIPHOSPHATE GLUCURONYLTRANSFERASE ACTIVITY BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN IN RATS

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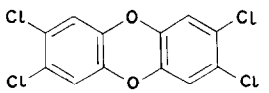
Abstract—After single low-level oral doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to rats, hepatic microsomal *p*-nitrophenol (PNP) glucuronyltransferase activity was elevated approximately 6-fold, whereas the hepatic glucuronyltransferase conjugating testosterone or estrone was unaffected. Solubilized and purified PNP glucuronyltransferase and steroid glucuronyltransferases from control and TCDD-treated rats exhibited the same relative activities (TCDD:control) as when the enzymes were bound to the endoplasmic reticulum. Elevation of PNP glucuronyltransferase was still evident 73 days after a single oral dose of 25 μg TCDD/kg. Female rats were more susceptible to TCDD actions on liver microsomal PNP glucuronyltransferase than males. The effects of TCDD treatment on PNP glucuronyltransferase appeared to be related to increased amounts of liver enzyme for the following reasons: (1) K_m values for PNP and UDPGA were unchanged by TCDD treatments; (2) the magnitude of the TCDD-induced increase of PNP glucuronyltransferase activity was the same whether enzyme activity was measured in the presence or absence of Mg^{2+} or Triton X-100; (3) TCDD, when added *in vitro*, had no detectable effect on enzyme activity; (4) TCDD treatment of rats did not change total hepatic microsomal phospholipid or cholesterol contents; (5) pH optima were unaffected by TCDD treatment; (6) solubilization of enzyme was not accompanied by a change in the TCDD induction effect; and (7) actinomycin D appeared to block the initial phase of induction.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a possible contaminant of



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

the herbicide 2,4,5-trichlorophenoxyacetic acid, is extremely toxic [1], although the mechanism of its toxic action is not known. At a recent conference on chlorinated dibenzodioxins and dibenzofurans, it was reported that TCDD is an extremely potent stimulator of some hepatic microsomal enzymes [2,3], including UDP glucuronyltransferase [3]. Glucuronyltransferases are components of the endoplasmic reticulum, and they function in the metabolism and excretion of xenobiotics. These enzymes also play a role in the metabolic regulation of many steroid compounds [4].

The sensitivity of hepatic microsomal enzymes to TCDD tissue levels was reflected by a doubling of *p*-nitrophenol (PNP) glucuronidation rates after a single oral dose of 0·2 μg TCDD/kg to female rats [3]. The stimulatory effect was still evident 38 days after TCDD treatment.

The present study was undertaken to characterize the nature of TCDD enhancement of UDP glucuronyltransferase. These studies include effects of divalent cations and detergents on liver microsomal glucuronyltransferase from control and TCDD-treated rats, determination of kinetic parameters, purification of glucuronyltransferases that conjugate steroid and non-steroid substrates from control and TCDD-treated rats, microsomal phospholipid and cholesterol determinations, the influence of age on TCDD actions, and attempts to block the induction of glucuronyltransferase using actinomycin D.

MATERIALS AND METHODS

Animals and preparation of subcellular fractions. Male and female rats (Charles River, CD strain) were used in these experiments. On the day of treatment, rats weighed approximately 200 g (males, 7-weeks-old; females, 8-weeks-old). In other experiments, 3-, 12- and 48-week-old male rats were used. TCDD was administered as a single oral dose in 0·4 ml acetone–corn oil (1:9) and controls received 0·5 ml acetone–corn oil. In some cases, actinomycin D was administered i.p. to male rats in 0·5 ml saline (1·0 mg/kg) immediately after TCDD treatment. Rats were killed by decapitation, livers removed, and microsomes prepared as described

previously [3]. Microsomes were washed once and resuspended in 1·15% KCl buffered with 0·02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7·5 at 5°) so that 1·0 ml microsomal suspension contained material from 0·5 g liver wet weight.

Glucuronyltransferase purification. Livers were homogenized in 0·25 M sucrose buffered with 50 mM Tris (pH 7·8) at 4°. Microsomes were prepared [3], washed once with buffered sucrose and finally resuspended in buffered sucrose so that 1·0 ml suspension contained material from 1·0 g liver wet weight. Glucuronyltransferase was solubilized by making the microsomal suspension 0·2 per cent with respect to sodium deoxycholate (DOC). This concentration of DOC was optimal for solubilization of glucuronyltransferase activity using either PNP or testosterone as the substrate. After centrifugation at 40,000 *g* for 45 min, the supernatant was dialyzed against three changes of 50 mM Tris containing 10 mM EDTA, and 0·2 mM mercaptoethanol (TEM) over a period of 24 hr. The dialyzed material was made 37 per cent with respect to ammonium sulfate, stirred 1 hr, and centrifuged at 30,000 *g* for 40 min. The precipitate was taken up in one-half the previous volume of TEM, and dialyzed for 16 hr against two changes of TEM. Dialyzed material (5·0 ml) was concentrated to 2·0 ml in dialysis tubing placed in Sephadex G-200. One ml of the concentrated fraction, containing material from 5·0 g liver (wet wt), was placed on a Sephadex G-200 column (25 mm × 45 cm, bed volume 176 ml) and 4·0-ml fractions were eluted at a flow rate of 14 ml/hr. TEM was the resolving buffer. Glucuronidation rates of testosterone, estrone and *p*-nitrophenol, and protein concentrations were determined for each fraction. The void volume was 67 ml as determined by elution of blue dextran (mol. wt = 2×10^6). Resolution of glucuronyltransferase on Sepharose (60–250 μm bead size, exclusion limit 10×10^6) was accomplished using the same procedure as that used for purification on Sephadex G-200.

Enzyme measurements. Glucuronidation of PNP and 1-naphthol, using approximately 0·5 mg protein from Triton X-100-treated microsomes, was determined as described previously [5,6] using an incubation time of 5 min. β-Glucuronidase was measured by determining the hydrolysis rate of PNP β-D-glucuronide [7]. Glucuronidation of testosterone and estrone was measured by a slightly modified method of Rao and Breur [8]. The incubation medium contained 0·3 mM testosterone or estrone, 1×10^5 dis/min ^{3}H-1,2-testosterone, ^{14}C-4-testosterone or ^{3}H-6,7-estrone, 0·8 mM UDPGA, 10 mM $MgCl_2$, and approximately 1·0 mg protein from Triton X-100-activated microsomes [6] in 1·0 ml of 75 mM Tris buffer (pH 7·5 at 37°) containing 50 μl propylene glycol. The reaction was stopped after 15 min by the addition of 5·0 ml ethyl acetate. Reaction rates were linear with respect to time for 45 min and linear with respect to the addition of microsomal protein up to 1·5 mg. When measuring glucuronyltransferase activity at each purification step, Triton X-100 was omitted from the reaction medium. The reason for omitting Triton X-100 was that the DOC, which was used to achieve solubilization, also activated glucuronyltransferase, and the addition of Triton X-100 to preparations previously treated with DOC resulted in lower enzyme activity than when either was used alone.

Phospholipid and cholesterol were extracted from microsomes with chloroform–methanol (2:1) [9], and

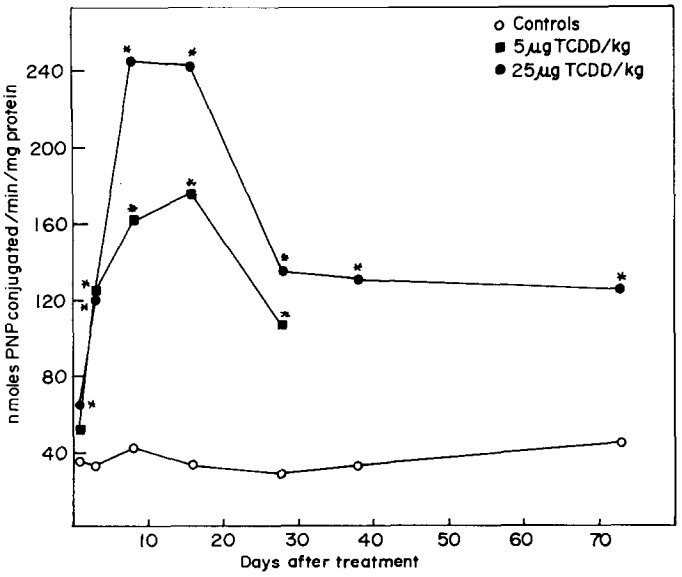


Fig. 1. Time-course effects of a single oral dose of TCDD on male rat liver microsomal glucuronyltransferase. N = three rats. An asterisk indicates that values are significantly different from controls at least at $P < 0{\cdot}05$. The first point represents day 1.

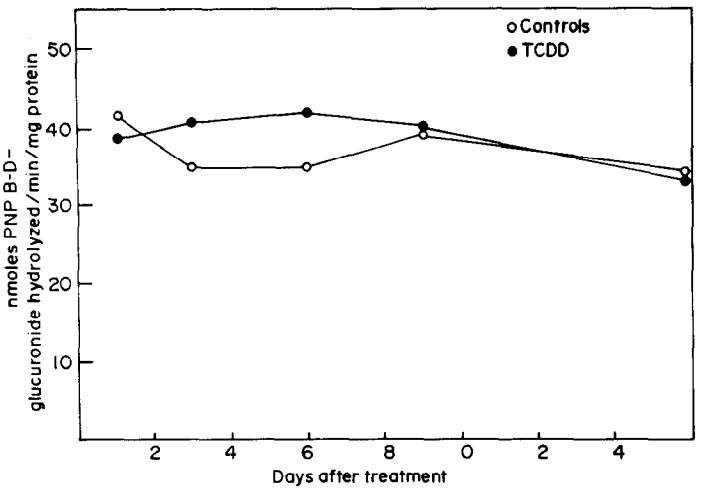


Fig. 2. Time–course effects of a single oral dose of 25 μg TCDD/kg on male rat liver microsomal β-glucuronidase. N = three rats. No values were significantly different from controls.

the phospholipid [10] and cholesterol [11] contents of the chloroform fraction determined. Microsomal protein contents were determined by the method described previously [12].

Validities of the assays for measuring PNP and testosterone glucuronidation rates were checked on DEAE-cellulose columns [13]. ^{14}C-ring-PNP (1·76 mCi/m-mole, 1 × 10^5 dis/min) was added to the incubation mixture with the usual quantity (100 μg) of unlabeled PNP. Other than the addition of ^{14}C-substrate to the PNP incubations, testosterone and PNP glucuronidation reactions were conducted as usual. After incubation, reaction mixtures were frozen for 2 weeks, thawed at room temperature and 1 ml of each sample was chromatographed on DEAE-cellulose columns [13]. Six-ml fractions were collected at a flow rate of 20 ml/hr. Unreacted PNP or testosterone eluted in the void volume, and these substrates were easily separated from their respective glucuronides. Quantification of ^{3}H and ^{14}C was accomplished by liquid scintillation. Unlabeled PNP was detected by measuring absorbance of each fraction at 405 nm, and unlabeled PNP β-D-glucuronide was detected by absorbance at 312 nm [6]. Chromatographic positions of ^{14}C-PNP, PNP β-D-glucuronide, ^{3}H-testosterone and ^{3}H-testosterone β-D-glucuronide standards were located on separate columns. Standard radiolabeled aglycones were resolved from their respective glucuronides when placed together on DEAE-cellulose columns.

RESULTS

Time–course and dose–response relationships. Male rats were given TCDD as a single oral dose at 5 or 25 μg/kg and PNP glucuronidation was measured 1, 3, 9, 16, 28, 38 and 73 days after treatment. The ED_{50} value for a single oral dose of TCDD is approximately 100 μg/kg.* Results for the time–course studies are illustrated in Fig. 1. After TCDD treatment at 25 μg/kg, enzyme activity was increased by 51 per cent on day 1, 162 per cent on day 3, 565 per cent on day 9, 636 per cent on day 16, 154 per cent on day 28, 162 per cent on day 38 and 160 per cent on day 73. Time–course effects were similar at the 5 and 25 μg TCDD/kg doses, although only the effects of the higher dose were studied 38 and 73 days after treatment. Elevation of 1-naphthol glucuronidation rates by TCDD pretreatment to rats was similar in magnitude to increases observed for PNP glucuronidation. Glucuronidations of ^{14}C- or ^{3}H-testosterone and ^{3}H-estrone by liver microsomes were not affected 1, 3, 6, 38 or 73 days after 25 μg TCDD/kg was given to rats.

β-Glucuronidase hydrolysis of PNP β-D-glucuronide, the product of PNP glucuronidation, was measured in liver microsomes 1, 3, 6, 9 and 16 days after rats received 25 μg TCDD/kg. These time–course studies revealed that deglucuronidation rates were not significantly changed (Fig. 2) in the same liver microsomes in which glucuronidations were markedly enhanced.

Influence of age on the response of PNP glucuronidation to TCDD. Male rats of different ages (17, 38, 80 and 335 days) were given a single oral TCDD dose and sacrificed 6 days after treatment. Results presented in Table 1 indicate that rat liver PNP glucuronidation is enhanced by approximately the same factor (500 per cent) in both mature and immature male rats. Enzyme activity in controls was highest in the 38- and 80-day-old rats.

DEAE-cellulose chromatography of incubation mixtures. To determine if the observed effects of TCDD were related to artifacts in the incubation medium, products were resolved from substrates on DEAE-cellulose columns. Representative elution profiles of

* John A. Moore, personal communication.

Table 1. Influence of animal age on TCDD-induced effects on male rat liver glucuronyltransferase

	p-Nitrophenol conjugated (nmoles/min/mg protein) Age (days) at treatment*			
	17	38	80	335
Control	20·5 ± 3·9	34·5 ± 7·3	39·1 ± 5·0	24·7 ± 1·9
TCDD-treated	112·0 ± 27·4	201·0 ± 19·8	190·6 ± 22·3	168·7 ± 30·4
TCDD-treated:control	5·5	5·8	4·9	6·8

* Rats were killed 6 days after treatment with a single oral dose of TCDD. 25 μg/kg. Each value represents the mean ± standard deviation derived from four animals.

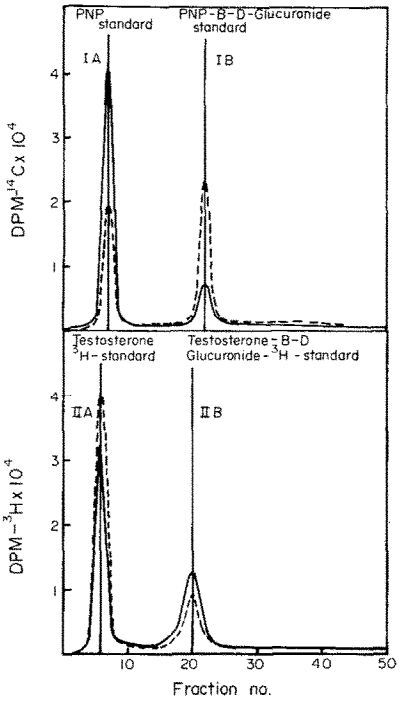


Fig. 3. DEAE-cellulose gradient elution profiles of microsomal incubations for measuring ^{3}H-testosterone or ^{14}C-PNP glucuronidation. The elution buffer started with 0·1 M Tris–HCl (pH 7·5) and ended with 0·05 M Tris–HCl (pH 7·5) with a total volume of 600 ml. Incubation mixtures were as described in Materials and Methods. Peak IA represents PNP, peak IB PNP β-D-glucuronide, peak IIA testosterone, and peak IIB testosterone β-D-glucuronide. Key: (——) incubations using liver microsomes from control rats; (– – – –) incubations using microsomes from TCDD-treated rats. Vertical solid lines represent chromatographic locations of standards as indicated.

Table 2. Quantification of glucuronyltransferase activity in hepatic microsomes from control and TCDD-treated rats*

Enzyme activity measured by	Testosterone glucuronidation (nmoles/min/mg protein) Control	TCDD	% Change	PNP glucuronidation (nmoles/min/mg protein) Control	TCDD	% Change
DEAE-cellulose chromatography†	4·75	3·40	−28	37·0	225·7	+510
Standard assays‡	3·71 ± 0·77	2·91 ± 0·93	−21	39·5 ± 2·3	253·9 ± 40·5	+543

* Male rats were given 25 μg TCDD/kg and sacrificed 4 days after treatment.

† Microsomal incubations from four rats pooled prior to chromatography.

‡ Testosterone glucuronidation was measured by extraction of testosterone β-D-glucuronide, and PNP glucuronidation was measured by PNP disappearance as described in Materials and Methods. Each value represents the mean ± standard deviation derived from four animals.

Table 3. TCDD effects on glucuronyltransferase activity in Triton X-100-activated, Mg^{2+}-activated and unactivated microsomes*

Substrate and animals	Substrate conjugated (nmoles/min/mg protein)†			
	$+MgCl_2$ +Triton	$-MgCl_2$ +Triton	$+MgCl_2$ −Triton	$-MgCl_2$ −Triton
PNP				
Control	40·5 ± 7·3	15·1 ± 0·6	3·7 ± 1·5	1·4 ± 0·5
TCDD	248·5 ± 43·4	76·4 ± 12·2	20·5 ± 1·9	7·1 ± 1·7
TCDD:control	6·1	5·1	5·5	5·1
Testosterone				
Control	5·4 ± 0·9	2·2 ± 0·3	0·7 ± 0·2	0·2 ± 0·1
TCDD	4·9 ± 0·3	2·1 ± 0·4	0·5 ± 0·2	0·2 ± 0·1
TCDD:control	0·9	1·0	0·7	1·0

* Male rats were sacrificed 6 days after a single oral dose of 25 μg TCDD/kg.
† Each value represents the mean ± standard deviation derived from three rats.

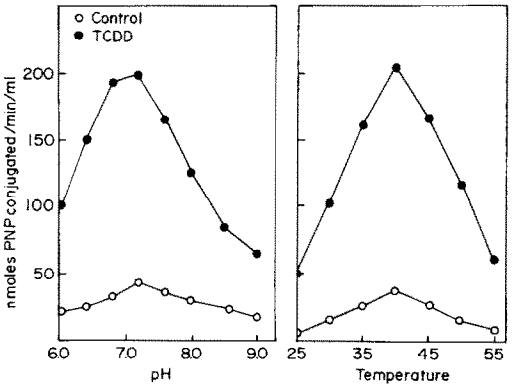


Fig. 4. pH and temperature optima for microsomal PNP glucuronidation. Each value represents an average of four incubations using liver microsomes from four control or TCDD-treated rats (25 μg TCDD/kg) sacrificed 6 days after treatment. Temperature optima were determined at pH 7·4, and pH optima were determined at 37°.

incubation mixtures for ^{3}H-testosterone or ^{14}C-PNP glucuronidations are illustrated in Fig. 3. Results from these experiments verify that the observed enhancement of liver microsomal PNP glucuronidation after TCDD pretreatment as measured by colorimetric procedures represents a real increase in enzyme activity (Table 2). Increases in PNP glucuronidations were 543 per cent when measured by PNP disappearance colorimetrically and 510 per cent when quantified by PNP β-D-glucuronide appearance on DEAE-cellulose columns. These data also verify that TCDD pretreatment does not increase liver microsomal testosterone glucuronidation (Table 3). Decreases in testosterone glucuronidations were 21 per cent when measured by the standard method and 28 per cent when quantified on DEAE-cellulose columns.

Studies on the mechanism of enhancement of PNP glucuronidation. Addition *in vitro* of TCDD (10^{-6} M) directly to microsomal incubations had no effect on glucuronidation rates of PNP, testosterone or estrone, indicating that TCDD actions on glucuronyltransferase were indirect. To further explore this idea, a number of experiments were done and are described as follows.

TCDD effects on glucuronyltransferase were determined in the presence and/or absence of Mg^{2+} and the detergent Triton X-100 (Table 3). Triton X-100 treatment (0·2 μl/mg of protein) of rat liver microsomes from either TCDD-treated or control rats increased PNP and steroid glucuronidation rates approximately 10-fold. Stimulation by Mg^{2+} of hepatic enzyme prepared from either control or TCDD-treated rats was approximately 2·5-fold. Thus, PNP glucuronidation was increased 5- to 6-fold by TCDD pretreatment to rats whether or not their subsequently isolated microsomes were treated with Triton X-100, or incubations performed in the presence or absence of Mg^{2+}. On the other hand, steroid glucuronidations were unaffected by TCDD regardless of these changes in the incubation medium. The pH and temperature optima are illustrated in Fig. 4. The pH optima were 7·2 for liver enzyme from either control or TCDD-treated rats and temperature optima were 40° for both sets of microsomal preparations. Kinetic parameters for glucuronyltransferase are illustrated in Table 4. These data reveal that the K_m for substrate (PNP) or cofactor (UDPGA) was not significantly affected by TCDD treatment (5 or 25 μg TCDD/kg), although marked increases in V_{max} were observed. Kinetic parameters were similar using enzyme from control or TCDD-treated rats whether activity was measured by PNP disappearance or PNP β-D-glucuronide appearance (Table 4).

Microsomal cholesterol and phospholipid contents were measured in control or TCDD-treated rats to determine if alterations in endoplasmic reticulum lipid content might be related to the observed effects on glucuronyltransferase. Data presented in Table 5 show that total microsomal cholesterol and phospholipid levels were not changed by TCDD treatment.

The above data suggest that TCDD-induced increases in PNP glucuronyltransferase are related to increased enzyme synthesis and/or decreased degrada-

Table 4. Kinetic properties of rat liver microsomal glucuronyltransferase after a single oral TCDD dose

Kinetic parameter	Dose (μg TCDD/kg)* 0	5	25
PNP disappearance			
K_m PNP†	0·26 ± 0·03	0·28 ± 0·02	0·26 ± 0·03
K_m UDPGA†	0·58 ± 0·15	0·73 ± 0·07	0·64 ± 0·08
V_{max}‡	126 ± 24	401 ± 32	539 ± 90§
PNP β-D-glucuronide appearance			
K_m PNP†	0·24 ± 0·03		0·29 ± 0·06
K_m UDPGA†	0·72 ± 0·05		0·61 ± 0·20
V_{max}‡	117 ± 23		548 ± 55§

* Rats were sacrificed 6 days after TCDD treatment. Each value represents the mean ± standard deviation derived from three male rats.

† K_m PNP was determined using 1·0 mM UDPGA. K_m UDPGA was determined using 0·8 mM PNP. K_m values are expressed in mM.

‡ V_{max} is expressed in nmoles PNP conjugated/min/mg of protein.

§ Significantly different from controls at $P < 0·01$.

Table 5. Male rat liver microsomal protein, phospholipid and cholesterol levels*

Animals	No. of animals	Protein (mg/g liver)	Phospholipid (mg/mg protein)	Cholesterol (μg/mg protein)
Control	4	22·1 ± 3·4	0·56 ± 0·04	31·4 ± 1·9
TCDD-treated	4	22·2 ± 4·5	0·55 ± 0·02	30·1 ± 1·3

* Values were measured 3 days after a single oral dose of 25 μg TCDD/kg.

Table 6. Effect of actinomycin D on hepatic glucuronyltransferase activity in control and TCDD-treated rats*

Animals	PNP conjugated (nmoles/min/g liver) −Actinomycin	+Actinomycin	PNP conjugated (nmoles/min/mg protein) −Actinomycin	+Actinomycin
Control	1066 ± 288	1078 ± 218	35·8 ± 11·0	55·8 ± 4·7
TCDD	1662 ± 60†	1110 ± 137	58·3 ± 3·6†	58·2 ± 4·0
TCDD:control	1·6	1·0	1·6	1·0

* Male rats were given 25 μg TCDD/kg orally followed immediately by 1·0 mg actinomycin D/kg, i.p. Rats were sacrificed 24 hr after treatment and each value represents the mean ± standard deviation derived from four rats.

† TCDD group significantly different from controls at least at $P < 0·05$.

tion. To test this hypothesis, control and TCDD-treated rats were given actinomycin D in an attempt to prevent increased glucuronyltransferase activity by inhibiting protein synthesis. Results were not conclusive, due to actinomycin D effects on total microsomal protein, but suggest that actinomycin D does block glucuronyltransferase induction (Table 6).

Purification of glucuronyltransferase from control and TCDD-treated rats. Liver microsomal steroid- and PNP-glucuronyltransferase activities were both elevated 7- to 10-fold by treatment *in vitro* with Triton X-100 (0·2 μl/mg of protein) or DOC (0·4 μg/mg of protein). However, Triton X-100 solubilized (centrifugal criteria; 105,000 *g* supernatant) only 28 per cent of PNP glucuronyltransferase compared to 71 per cent after DOC treatment. For this reason, we selected DOC as the solubilizing agent in our attempts to purify glucuronyltransferase. DOC must be immediately removed (at least excess DOC) after solubilization, since storage of DOC-treated microsomes in solutions still containing excess DOC resulted in almost complete loss of glucuronyltransferase activity (Fig. 5).

The purification procedure and recoveries at each step for male liver glucuronyltransferase catalysis of testosterone, estrone and PNP conjugations are outlined in Table 7. Approximately 6-fold elevations of PNP glucuronidation rates were observed by TCDD at each purification step, whereas steroid glucuronidation rates, using enzyme from control or TCDD-treated rats, were similar throughout the purification procedure. Testosterone glucuronidation rates *in vitro* were approximately $\frac{1}{8}$ those for PNP glucuronidation,

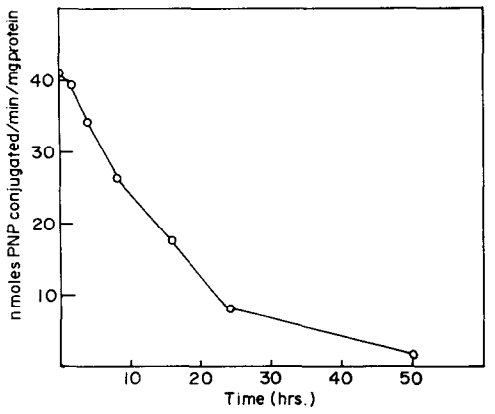


Fig. 5. Loss of liver microsomal PNP glucuronyltransferase activity on storage of microsomes at 4° in the presence of DOC (0·4 μg/mg of protein).

although recoveries of the respective glucuronyltransferases were approximately the same (Table 7). The glucuronyltransferases that conjugate PNP, estrone and testosterone are eluted in the void volume from Sephadex G-200 columns, indicating molecular weights of greater than 500,000. Elution profiles, illustrated in Fig. 6, show that the highest activities occurred in fraction 17. Approximately 50 fractions were collected which included proteins of molecular weights as low as 12,000 as determined by marker proteins (aldolase, ribonuclease A and chymotrypsinogen A). Only one enzyme peak was resolved, although several protein peaks were detected by absorbance profiles at 254 and 280 nm. This purification procedure yielded an increase in specific activity of approximately 100 above that of unactivated microsomes using PNP, testosterone or estrone as the substrate. The increase in specific activity was approximately 10-fold higher than preparations from DOC-activated microsomes.

In some experiments, Sepharose 4B (exclusion limit 10×10^6, bead size 60–250 μm) was employed to resolve glucuronyltransferase rather than Sephadex G-200 due to the high molecular weight of the enzyme and the high exclusion limit of Sepharose. Results from these experiments are presented in Fig. 7. Two activity peaks were detected for glucuronyltransferase whether PNP or testosterone was used as the substrate, and the chromatographic locations of testosterone glucuronyltransferase were identical to those for PNP glucur-

Table 7. Purification procedure and recoveries of rat liver glucuronyltransferase from control and TCDD-treated rats*

Substrate and purification step	Controls		TCDD-treated (25 μg/kg)	
	Sp. act.	Recovery (%)	Sp. act.	Recovery (%)
PNP glucuronidation				
Microsomes	3·5		21·2	
DOC-treated microsomes	29·2	100	200·5	100
DOC supernatant	43·3	91	283·0	86
Ammonium sulfate fraction (0·37 ppt)	66·4	63	420·9	65
Sephadex G-200 fraction 17	307·4	35	1804·7	38
Sepharose fraction 15	296·4	24	1422·3	22
Sepharose fraction 24	627·8	9	2930·5	9
Testosterone glucuronidation				
Microsomes	0·41		0·31	
DOC-treated microsomes	3·05	100	2·48	100
DOC supernatant	4·46	91	4·59	113
Ammonium sulfate fraction (0·37 ppt)	6·60	59	6·01	79
Sephadex G-200 fraction 17	37·93	41	42·14	64
Sepharose fraction 15	26·15	27	24·36	32
Sepharose fraction 24	69·02	13	61·15	17
Estrone glucuronidation				
Microsomes				
DOC-treated microsomes	0·20	100	0·17	100
DOC supernatant	0·27	73	0·26	102
Ammonium sulfate fraction (0·37 ppt)	0·43	64	0·41	81
Sephadex G-200 fraction 17	2·07	35	2·48	53

* Purification procedure and assay methods are described in Materials and Methods. Specific activity is expressed in nmoles substrate conjugated/min/mg of protein. Each test group was derived from pooled livers from four male rats. Rats were sacrificed 6 days after 25 μg TCDD/kg. Recoveries are percentage of total activity detected in DOC-activated microsomes.

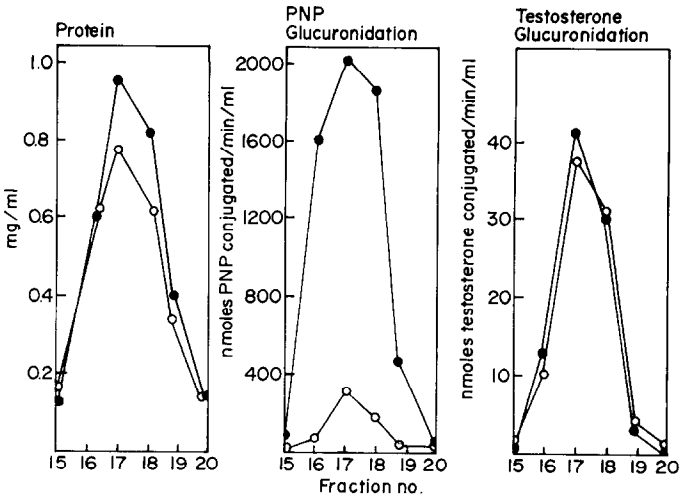


Fig. 6. Elution of the concentrated dialyzed ammonium sulfate fraction containing glucuronyltransferase on Sephadex G-200. Fifty 4·0-ml fractions were collected, but only fractions 15–20 are illustrated, since they were the only fractions containing enzyme activity. The void volume was eluted by fraction 17. Key: (O——O) preparations from control rats; (●——●) preparations from TCDD-treated (25 μg/kg single dose) rats.

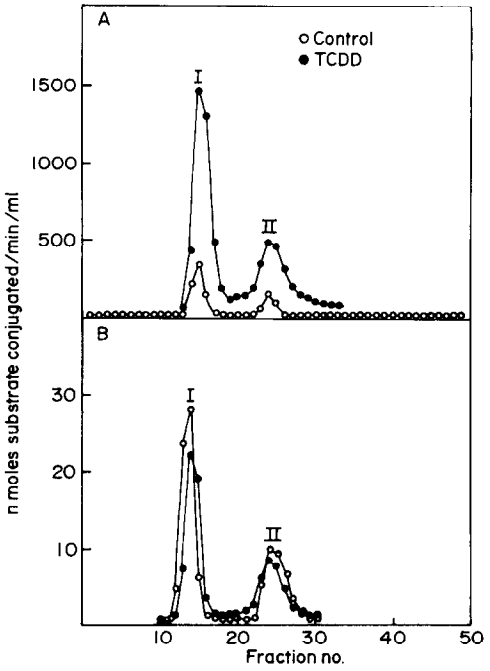


Fig. 7. Elution of the concentrated dialyzed ammonium sulfate fraction containing glucuronyltransferase on Sepharose 4B. Sixty 4·0-ml fractions were collected. Key: (O——O) preparations from control rats; (●——●) preparations from TCDD-treated (25 μg/kg single dose) rats. Panel A represents the elution profile of the glucuronyltransferase conjugating PNP, and panel B represents elution of the glucuronyltransferase conjugating testosterone.

onyltransferase. Hepatic enzyme preparations from TCDD-treated rats eluted at the same chromatographic locations as did preparations from control animals (Fig. 7), although specific activities for PNP glucuronidations were much higher in preparations from the TCDD-treated group. Activity peak I from control animals contained 72 per cent of the total PNP glucuronyltransferase activity eluted from Sepharose columns, and the remaining 28 per cent eluted in peak II. The respective peaks from TCDD-treated rats contained 69 per cent (peak I) and 31 per cent (peak II) of the total activity. Percentages of total eluted testosterone glucuronyltransferase were: control peak I, 68 per cent; control peak II, 32 per cent; TCDD peak I, 65 per cent; TCDD peak II, 35 per cent. The increase in specific acitivity of PNP glucuronidation rates of peak II above the rates for unactivated microsomes was approximately 150. After centrifugation of fractions from peak I at 105,000 *g* for 8 hr, essentially all the enzyme activity was detected in the pellet, whereas after similar centrifugation of peak II, enzyme activity remained in the supernatant. These data indicate that peak II represents solubilized enzyme, whereas peak I contains glucuronyltransferase bound to fragments of the endoplasmic reticulum.

DISCUSSION

These studies characterize and validate the sensitivity of hepatic microsomal PNP glucuronyltransferase to TCDD body burdens. A singe oral dose of 1·0 μg TCDD/kg to male rats ($LD_{50} \cong 100$ μg TCDD/kg) significantly elevated liver microsomal PNP glucuronidation rates *in vivo* [3]. On the other hand, TCDD

had no detectable effect on deglucuronidation as catalyzed by liver microsomal β-glucuronidase. Therefore, net glucuronidation in liver should be substantially increased by TCDD. Elevation of glucuronyltransferase activity by TCDD is approximately 100,000 times more potent on a μg/kg basis than that observed for the classical inducing agents, 3-methylcholanthrene and phenobarbital [14,15]. Female rats have been shown to be more susceptible to TCDD-induced effects on hepatic microsomal PNP glucuronyltransferase than male rats, and a marked elevation in enzyme activity was still evident in both sexes 38 days after a single oral dose of 25 μg TCDD/kg [3]. Increased activity of some rat liver mixed function oxidase components was also observed 73 days after 25 μg TCDD/kg [16]. The long duration of TCDD actions on hepatic microsomal components might be related to the concentration of TCDD in the hepatic endoplasmic reticulum [17].

The mechanism of TCDD elevation of hepatic glucuronyltransferase remains unclear, although the action appears to be indirect, since TCDD had no detectable effect on PNP glucuronidation rates when added *in vitro* to microsomal preparations. TCDD apparently does not enhance glucuronyltransferase activity by alterations in kinetic parameters, since K_m values for PNP and UDPGA were unchanged by TCDD treatment. Divalent cations and many detergents are activators of microsomal glucuronyltransferase *in vitro* [6], and TCDD actions on hepatic PNP glucuronyltransferase were thought to be possibly related to a detergent-type effect on hepatic microsomes or to a mobilization of endogenous magnesium. However, data reported here demonstrate that the magnitude of the increase of PNP glucuronyltransferase was the same whether enzyme activity was measured in the presence or absence of Mg^{2+} or Triton X-100. Microsomal glucuronyltransferase activity is phospholipid dependent [18, 19], and microsomal cholesterol might function in the maintenance of endoplasmic reticulum structure [20]. However, TCDD-induced alterations in PNP glucuronyltransferase activity do not appear to be related to effects on total microsomal phospholipid or cholesterol, although effects on individual phospholipids have not been studied. These data suggest that TCDD enhancement of PNP glucuronyltransferase is not related to alterations in endoplasmic reticulum structure, although this possibility has not been entirely precluded. At this stage, however, the possibilities of increased enzyme synthesis and/or decreased rate of degradation are more probable explanations. TCDD induction of aminolevulinic acid (ALA) synthetase in chick embryo was blocked by cycloheximide [21], but effects of protein-synthesis blocking agents are difficult to study in rats due to the lag period in PNP glucuronyltransferase induction and the rapid toxicity of antimetabolites. Experiments done in this laboratory were inconclusive but indicated that actinomycin D does block glucuronyltransferase induction by TCDD, lending further credence to the idea that TCDD-induced increases in enzyme activity are a factor of increased enzyme synthesis.

For the following reasons, the observed increase in hepatic PNP glucuronyltransferase activity after TCDD treatment to rats appears to represent an increase in enzyme activity rather than artifacts in the incubation medium: (1) enhancement of PNP glucuronidation was about 6-fold at each purification step, including solubilization, indicating that TCDD actions on glucuronidation rates are not related to alterations in membrane environment, although centrifugal criterion alone is not sufficient to prove true solubilization [22]; (2) elevation of enzyme activity, 6 days after a single oral dose of 25 μg TCDD/kg, was approximately 6-fold whether enzyme activity was measured by PNP disappearance or PNP β-D-glucuronide appearance; (3) elevation of enzyme activity was approximately 6-fold whether 1-naphthol (radioactive assay) or PNP was used as the substrate; and (4) elevations of PNP glucuronidation rates were approximately 6-fold whether enzyme activity was measured by resolving substrate from product on DEAE-cellulose columns or measured by colorimetric methods.

Surprisingly, the hepatic microsomal glucuronyltransferase conjugating testosterone or estrone was unaffected by TCDD treatment in the same animals that a marked increase of PNP glucuronyltransferase was detected. The lack of an observed effect on steroid glucuronidation is not related to limitations in the assay method, since slight decreases in enzyme activity were detected in hepatic microsomes from TCDD-treated rats whether glucuronidation was measured by the standard method [8] or by quantification of product on DEAE-cellulose columns. The different effect of TCDD on steroid glucuronidation compared to PNP and 1-naphthol glucuronidations provides further evidence for the theory that there is more than one liver microsomal glucuronyltransferase [23, 24]. PNP or steroid glucuronyltransferase exhibited similar recoveries at each purification step, similar chromatographic properties on Sepharose columns, and similar activations by Triton X-100, DOC and Mg^{2+}, indicating that the physical properties of these glucuronyltransferases are similar.

The lack of effect of TCDD on steroid glucuronidations suggests that the marked increases in PNP glucuronidation rates do not necessarily mean that the normal regulation of steroid hormones will be disrupted by enhancing their rate of conjugation and subsequent excretion. However, it should be pointed out that testosterone hydroxylations are affected by TCDD treatment [16], thus indirectly affecting conjugation rates *in vivo*.

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