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Characterization of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Mediated Decreases in Dexamethasone Binding to Rat Hepatic Cytosolic Glucocorticoid Receptor

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SUMMARY

An investigation of the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the liver cytosolic glucocorticoid receptor (GRc) in intact and adrenalectomized (ADX) rats, using equilibrium binding analysis, sucrose gradient sedimentation, and affinity labeling experiments, clearly demonstrated that TCDD significantly reduced the binding capacity (B_{max}) of the hepatic GRc but did not alter the apparent equilibrium dissociation constant (K_d). This effect was maximal after 24 hr and was still present 22 days after treatment. Western blot analysis revealed that TCDD treatment did not cause a comparable decrease in the levels of immunodetectable receptor protein, which suggests that the steroid-binding properties of the hepatic GRc are altered, rather than the absolute concentration of receptor protein. Studies of TCDD effects on the uptake of GRc by nuclei indicated that TCDD treatment did not alter the ability of the steroid-GRc complex to be taken up by nuclei; however, TCDD treatment did increase the total capacity of liver nuclei to bind steroid-GRc complexes. TCDD dose-response studies that compared the hepatic GRc steroid binding of ADX and intact rats indicated that adrenalectomy markedly enhanced the response to TCDD. Significant effects on the GRc binding in ADX animals were induced at TCDD doses that were 10,000 times lower than those required for a response in intact rats. Analysis of two other biochemical markers demonstrated that ADX rats were 10-fold more sensitive to the induction of microsomal benzo[*a*]pyrene hydroxylase but of similar sensitivity to reduction of epidermal growth factor receptor binding, when compared with the responses of intact animals. These data indicate that adrenal status may be important in modulating the responses of the animals to TCDD and that the alteration of the hepatic GRc pathway may have a role in some of the actions of TCDD.

TCDD is an environmental contaminant formed during the synthesis of the herbicide 2,4,5-trichlorophenoxyacetic acid (1, 2). Accidental human exposure to TCDD has resulted in numerous clinical reports describing chloracne and hepatic dysfunction (3). In certain animal models, TCDD is a potent teratogen (3), hepatocarcinogen (4), and liver tumor promoter (5). In addition, it induces a number of hepatic enzymes such as microsomal BPH and it influences hepatic plasma membrane proteins such as the EGF receptor (6). Although the mechanism of TCDD-induced toxicity is not clear, the involvement of a specific intracellular protein, the Ah receptor, has been postulated to mediate some of the toxic effects of TCDD (7, 8). In addition to this receptor system, circulating glucocorticoids and/or the GRc pathway may also be involved in mediating TCDD toxicity because ADX rats are more sensitive to the lethal effects of TCDD than are intact animals (9–11).

In target tissues, the GRc is an intracellular steroid binding protein that specifically binds glucocorticoid-like steroids and undergoes a process known as "transformation" that results in the exposure of a DNA-binding site and enables the steroid-GRc complex to translocate to the nucleus, bind to chromatin, and alter transcription of specific genes (12, 13). The present report describes time course and dose-response relationships of TCDD-mediated effects on the hepatic GRc in intact and ADX adult female rats.

Materials and Methods

Chemicals. [1,2,4,6,7-^{3}H]Dexamethasone (70 to 90 Ci/mmol, radiochemical purity >96%) was purchased from Amersham/Searle (Arlington Heights, IL); [^{3}H]dexamethasone 21-mesylate (50 Ci/mmol) and Biofluor scintillation cocktail were bought from New England Nuclear-DuPont (Boston, MA). TCDD (99.9% purity by gas chromatography-mass spectrometry) was obtained as a gift from Dr. James McKinney (National Institute of Environmental Health Sciences, Re-

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ADX, adrenalectomized; GRc, cytosolic glucocorticoid receptor; BPH, benzo[*a*]pyrene hydroxylase; EGF, epidermal growth factor; dexamethasone, 1,4-pregnadien-9-fluoro-16α-methyl-11β,17α,21-triol-3-20-dione; SDS, sodium dodecyl sulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.

search Triangle Park, NC). [^{125}I]-labeled receptor grade EGF, with a specific activity of 100 to 200 μCi/μg, was prepared at Meloy Laboratories (Springfield, VA) by the chloramine-T method (14) and was purified by Sephadex G-10 column chromatography. Immunological reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Fisher (Raleigh, NC). Nitrocellulose paper (0.45 μm) and electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA). The BuGR2 monoclonal antibody was kindly provided by Dr. Robert W. Harrison (University of Arkansas Medical School).

Animals and treatments. Adult female Sprague-Dawley rats (60 days old; 200 to 250 g) were purchased from Charles River Laboratories (Kingston, NY). In some experiments, animals were adrenalectomized at Charles River Laboratories and were maintained for 10 to 13 days on 0.9% NaCl. Sham-operated controls were included in all experiments involving ADX animals. A single treatment of TCDD was administered, in corn oil, by oral gavage at doses of 0.1 to 25 μg/kg of body weight for the intact and from 0.1 ng/kg to 5 μg/kg for the 13-day ADX rats. Liver and body weights were used as markers of toxicity resulting from TCDD treatment. Administration of TCDD (0.1 to 25 μg/kg) to sham-operated intact rats significantly increased liver weights only at doses of TCDD greater than 10 μg/kg and had no significant effect on body weight, compared with the corn oil-treated animals. Although adrenalectomy resulted in a significant reduction in body weights compared with the controls, administration of TCDD (0.001 to 5 μg/kg) to ADX animals did not influence body or liver weights. As reported for other strains of ADX rats, our CD-1 ADX rats were found to be very sensitive to the lethal effects of TCDD. TCDD doses higher than 5 μg/kg were lethal (50–75% mortality) for our rats; therefore, we did not exceed this dose in ADX rats.

Preparation of hepatic subcellular fractions. For the preparation of cytosol, the liver was minced with scissors and a 3-g portion was homogenized (1:3, w/v) in an ice-cold TEDGM buffer that contained 10 mM Tris·HCl, 2.5 mM EDTA, 5 mM dithiothreitol, 10% (v/v) glycerol, and 20 mM sodium molybdate (pH 7.4), using a motor-driven, Potter-Elvejeheim homogenizer (10 strokes). The homogenate was centrifuged at 10,000 × g for 20 min, and the resulting supernatant was then recentrifuged at 105,000 × g for 65 min. The supernatant (containing cytosol) was carefully recovered by aspiration and stored in 2-ml aliquots at −70° until analysis. Microsomes were prepared separately following methodology similar to that for cytosol, described above, except that 1.15% KCl/50 mM Tris·HCl (pH 7.4) was used for the homogenization step. Each homogenate was centrifuged at 10,000 × g for 20 min, and the supernatant was then centrifuged at 105,000 × g for 65 min. The resulting pellet was then washed with 50 mM Tris·HCl (pH 7.4) before recentrifugation at 105,000 × g for 30 min. The supernatant was discarded, and the pellet was resuspended in a 10 mM HEPES/250 mM sucrose buffer (pH 7.6) before storage at −70°. For the nuclear binding experiments, liver cytosol and nuclei were prepared by the methods described by others (15, 16). Hepatic plasma membranes were prepared according to a modification of the method of Neville (17). Protein was quantified by the method of Bradford (18).

Receptor assays. The cytosolic glucocorticoid binding assay was carried out according to the methods of Rosner and Polimeni (19) and Beato and Feigelson (20). Specifically bound [^{3}H]dexamethasone was calculated as the difference between total and nonspecifically bound fractions for each cytosolic preparation. Data were fitted by the method of least squares, and the apparent equilibrium dissociation constant (K_d, in nM) and apparent maximal binding capacity (B_{max}, in fmol of [^{3}H]dexamethasone/mg of cytosolic protein) were estimated by Scatchard analysis (21). These assay conditions were linear with respect to protein from 1 to 5 mg/ml cytosol and were carried out under equilibrium conditions. To demonstrate that our assay conditions measured specific glucocorticoid binding, cytosol was incubated with 1 nM [^{3}H]dexamethasone in the absence or presence of various molar ratios (10- to 10,000-fold) of different unlabeled steroid competitors. These studies indicated that [^{3}H]dexamethasone specifically bound to the rat liver GRc and had a characteristic equilibrium dissociation constant and specificity.

Nuclear GRc uptake assay. This assay was performed using established methods (15, 16). DNA measurements were done using the fluorometric method of Downs and Wilfinger (22).

Sucrose and gradient ultracentrifugation. Fixed angle rotor gradients of 5 to 20% sucrose (w/v; total of 6 ml) were prepared in TEDGM buffer, according to protocols of Eastman-Reks *et al.* (23) and Griffiths (24), using Beckman polyallomer bell top quick-seal ultracentrifuge tubes (16 × 45 mm). Fractions (0.2 ml) were collected and assayed for radioactivity and protein content. The gradients were reproducible and were linear with density, as evidenced by increasing R_F values from 1.35 to 1.37. Sedimentation coefficients ($s_{20,w}$) of the hepatic [^{3}H]dexamethasone GRc complexes were also determined according to the method of Martin and Ames (25), using myoglobin (1.9 S), carbonic anhydrase (2.9 S), bovine serum albumin (4.6 S), aldolase (7.4 S), β-amylase (8.9 S), and catalase (11.2 S) as standards. These conditions consistently yielded linear standard curves in the range of 1.9 to 11.2 S and were reproducible between experiment days, with the GRc peak appearing between the 12th and 20th fractions. Sucrose gradient profiles of the GRc complexes shown in the figures are representative of three or more replicate experiments.

Affinity labeling of the hepatic GRc. Affinity labeling was carried out according to a modification of the protocol of Eisen *et al.* (26). Low molecular weight components in the liver cytosol that interfere with affinity labeling were removed by means of Sephadex G25M gel permeation chromatography, using disposable PD-10 columns (Pharmacia Fine Chemicals, Uppsala, Sweden). Appropriate column aliquots, containing approximately 300 μg of protein in a volume not more than 100 μl, were removed and treated with 1 ml of 10% trichloroacetic acid for 1 hr at 4°. Protein precipitates were collected by centrifugation at 10,000 × g for 10 min and were washed with acetone. The pellets were dissolved in SDS-polyacrylamide gel electrophoresis sample buffer (2% SDS, 0.0125% bromophenol blue, 4% glycerol (v/v), 1.25% mercaptoethanol, 10 mM Tris, pH 6.8), boiled for 3 min, and separated by electrophoresis on 8% SDS-polyacrylamide gels. Gels were stained using 0.01% Coomassie blue (R-250), prepared for fluorography by incubation in Enhance solution (NEN), dried, and subjected to autoradiography.

Immunoprecipitation and Western blot analysis of the rat hepatic GRc. In order to enhance the detection of the liver GRc and to reduce nonspecific background, the GRc was first immunoprecipitated from crude cytosol and then analyzed by immunoblotting. The BuGR2 monoclonal antibody prepared by Dr. Robert Harrison (27) was produced against the rat GRc and has been shown to react with an epitope in the DNA-binding domain of the receptor (28). Aliquots of rat hepatic cytosol were mixed with an equal volume of TEG buffer (10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, 50 mM NaCl, pH 7.6 at 4°). BuGR2 or nonimmune mouse IgG were added at 3% of the final volume and were incubated for 2 hr at 0–4°. Each sample was added to a 40-μl Protein A-Sepharose pellet (equilibrated in TEG buffer) and mixed by rotation for 2 hr at 4°, followed by washing of the Protein A-Sepharose pellets four times with 1 ml of TEG buffer. The pellets containing the immunoprecipitated GRc were then prepared for immunoblotting by resuspension in 2× SDS sample buffer, boiling for 5 min, and then resolving of the eluted proteins by electrophoresis on 7% SDS-polyacrylamide gels (29). Immunoblotting of the GRc was carried out by transferring proteins to nitrocellulose paper, followed by incubation for 3 hr with 3% BuGR2 monoclonal antibody and subsequent reaction with peroxidase-conjugated goat anti-mouse IgG, as previously described (30, 31).

EGF radioreceptor assay. ^{125}I-EGF receptor equilibrium binding constants were determined by following the modification of the protocol of Carpenter and Cohen (32). Specific EGF binding was calculated as the difference between total and nonspecific binding and was expressed as fmol of ^{125}I-EGF/mg of protein.

BPH assay. This fluorimetric assay was carried out according to

the method of Nebert and Gelboin (33). 3-Hydroxybenzo[*a*]pyrene was obtained from the National Cancer Institute Respository and was used as the standard. The BPH activities were expressed as pmol of 3-hydroxybenzo[*a*]pyrene formed/min/mg of microsomal protein.

Statistical analysis. Data were expressed as the mean ± standard error; the numbers in parenthesis denote the number of animals analyzed. Differences between control and test groups were analyzed using Student's *t* test (35). Differences were considered significant at $p < 0.05$.

Results

The effect of TCDD administration on the dexamethasone binding properties of the hepatic GRc was examined by both sucrose gradient profiles and the determination of the apparent [^{3}H]dexamethasone binding constants at equilibrium binding conditions. The sucrose gradient profiles (Fig. 1) of displaceable [^{3}H]dexamethasone-GRc binding complexes of hepatic cytosol prepared from intact (Fig. 1A) and ADX (Fig. 1B) female rats 10 days after treatment with corn oil or TCDD show similar sedimentation profiles, with a major peak at 8.2 ± 0.4 S and a minor peak at 4.9 ± 0.2 S ($n = 5$). TCDD treatment of intact (25 μg/kg) or ADX (2.5 μg/kg) rats resulted in significantly lower binding (>50%), compared with the corn oil-treated controls, but did not dramatically alter the sedimentation coefficients (7.9 ± 0.3 S and 5.0 ± 0.2 S).

Analysis of the apparent equilibrium binding constants (K_d and B_{max}) of the hepatic GRc demonstrated that TCDD treatment reduced [^{3}H]dexamethasone binding by approximately 50% of that obtained for controls; however, there was no apparent change in the glucocorticoid receptor equilibrium dissociation constant, K_d (Fig. 2). Fig. 2 represents a typical Scatchard-type plot, a specific and nonspecific dexamethasone binding plot (Fig. 2, *inset A*), and a Hill plot (36) (Fig. 2, *inset B*) of the hepatic GRc obtained for a corn oil- and a TCDD-treated intact rat. Replicate experiments demonstrated that TCDD treatment consistently decreased the maximal binding capacity (B_{max} = 137 ± 5 fmol/mg) of the hepatic GRc but did not have a dramatic effect on the apparent equilibrium dissociation constant (K_d = 0.52 ± 0.05 nM), when compared with the corn oil-treated controls (B_{max} = 307 ± 19 fmol/mg; K_d = 0.67 ± 0.04) ($n = 23$). Further analysis using the Hill plot (Fig. 2, *inset B*) (36) revealed that, for both control and TCDD-treated animals, the Hill coefficient (n_H) was approximately equal to unity (control, 1.00 ± 0.04; TCDD, 0.99 ± 0.03), which suggested that [^{3}H]dexamethasone was associating with a non-interacting single class of binding sites present in the hepatic cytosol. Similar to intact animals, analysis of the [^{3}H]dexamethasone binding to the hepatic GRc of ADX animals also revealed that TCDD treatment decreased the B_{max} of the receptor but had little effect on the K_d (data not shown).

Affinity labeling of crude rat liver cytosolic receptors *in vitro* using [^{3}H]dexamethasone 21-mesylate was used to further evaluate the effect of TCDD on the hepatic GRc. Autoradiography (Fig. 3) revealed three major proteins that were covalently labeled (M_r = 94,000, 66,000, and 50,000) in liver cytosolic samples incubated with [^{3}H]dexamethasone 21-mesylate alone. However, only the labeling of the M_r 94,000 protein was specifically displaced by incubation in the presence of excess unlabeled dexamethasone. The specific covalent labeling of the M_r 94,000 protein, which is the reported molecular weight of the GRc, provided evidence that this protein was the rat liver GRc (26, 31). The autoradiogram clearly demonstrated that TCDD treatment decreased the amount of GRc that was affinity labeled in ADX rats. Similar results were also obtained using cytosol of sham-operated control and TCDD-treated rats (data not shown).

The effects of TCDD on the hepatic GRc were also determined at various time periods after a single administration of TCDD (Fig. 4). TCDD (25 μg/kg) treatment of intact rats significantly decreased the maximum GRc binding capacity of hepatic GRc, by approximately 40% as early as 24 hr, and it remained maximally decreased as long as 22 days (B_{max} = 157 ± 20 fmol/mg), compared with the control (B_{max} = 289 ± 41 fmol/mg for the same experimental day).

Further studies also revealed that reduction in hepatic GRc binding was not due to the decrease in feeding elicited by TCDD administration. Although TCDD treatment (25 μg/kg; 10 days) reduced food intake by 16%, the GRc equilibrium binding constants were not altered in the pair-fed when compared with *ad libitum* fed rats (K_d in nM, B_{max} in fmol/mg of protein); fed *ad libitum* (0.85, 269); pair-fed (0.72, 303); and TCDD-treated (0.54, 174). The lack of depression in hepatic GRc levels in pair-fed, compared with the *ad libitum* fed ani-

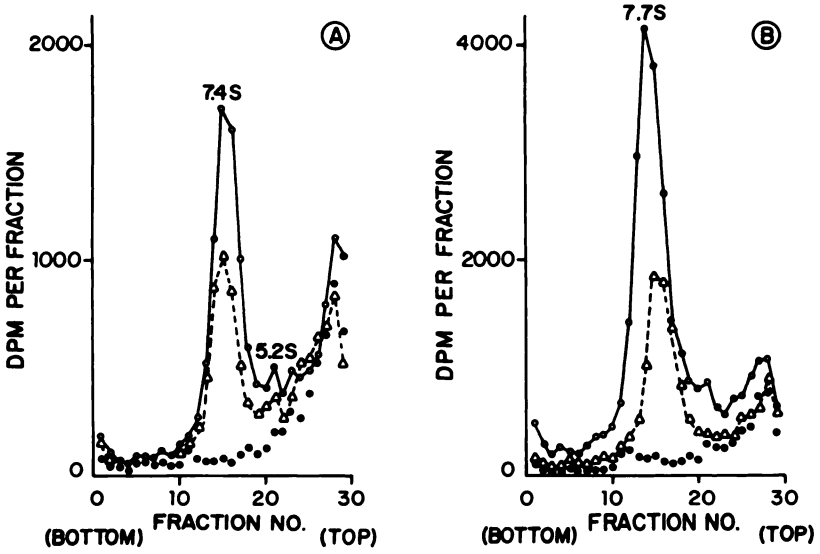


Fig. 1. Sucrose gradient profile of the hepatic glucocorticoid receptor binding of [^{3}H]dexamethasone following a single administration of corn oil or TCDD to intact (25 μg/kg) (A) and ADX (2.5 μg/kg) (B) adult female rats. A lower TCDD dose was used to treat the ADX rats because of the increased sensitivity of the ADX animals to the lethal effects of TCDD. Cytosols prepared from corn oil-treated controls (○) or TCDD-treated (△) animals were incubated with [^{3}H]dexamethasone in the presence (●) (nonspecific binding) or absence (○, △) (total binding) of unlabeled competitor, as described in Materials and Methods. Nonspecific binding (●) was similar for both the corn oil- and the TCDD-treated animals; therefore, only the data from corn oil-treated rats are shown.

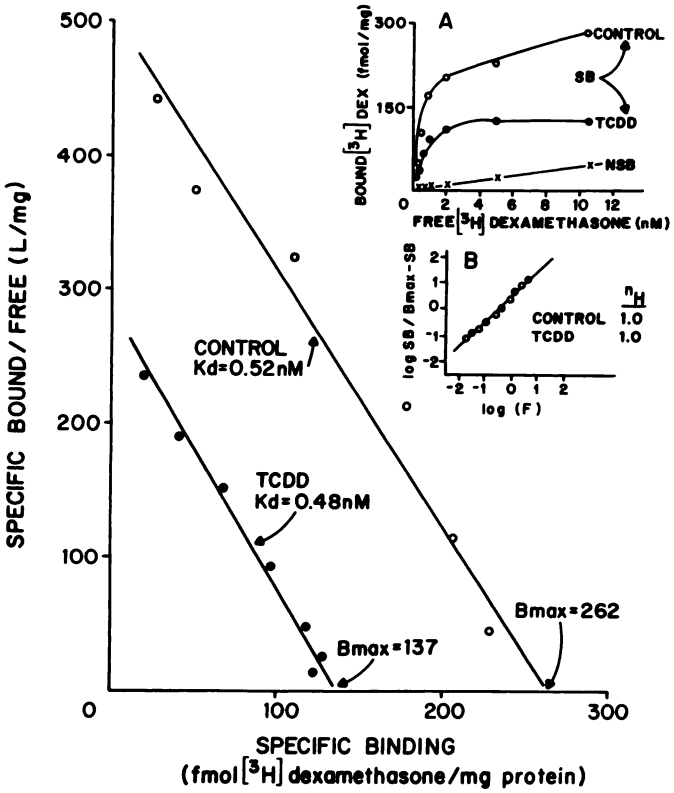


Fig. 2. Representative Scatchard plot of the hepatic GRc in adult intact female rats 10 days after treatment with a single dose (25 μg/kg) of TCDD (●) or the corn oil vehicle (○). *Inset A*, the specific (●, ○) and nonspecific (×) [^{3}H]dexamethasone binding data used for the Scatchard plot. Nonspecific binding for both control and TCDD-treated animals was linear with respect to the concentration of free hormone and represented 5 to 10% of total binding (*inset A*). In this figure, for the sake of simplicity, only the nonspecific binding values for the controls are shown, but similar values were obtained for the TCDD-treated rats. In all cases, specific binding was calculated from the individual total and nonspecific binding values for the respective TCDD-treated or control animals. TCDD treatment dramatically reduced the maximal binding capacity (B_{max}) of the hepatic GRc but did not significantly affect the apparent equilibrium dissociation constant (K_d). *Inset B*, the Hill plot for the hepatic GRc of the control (○) and the TCDD-treated (●) animals. A Hill coefficient (n_H) of unity suggests noninteracting sites of hepatic GRc.

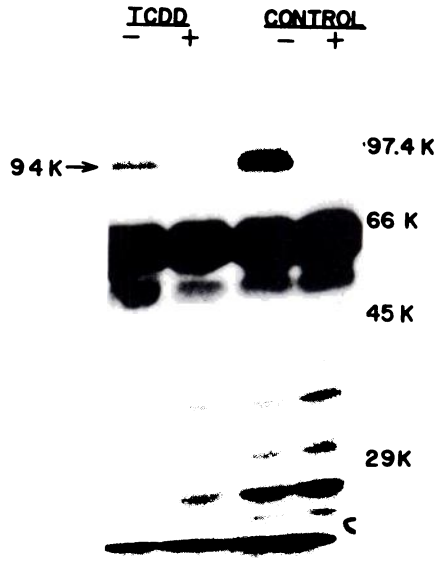


Fig. 3. Affinity labeling of the ADX rat hepatic GRc. This figure represents an autoradiogram of a gel demonstrating the covalent labeling of the cytosolic proteins in the presence (+) and absence (−) of excess unlabeled dexamethasone. Note that the affinity labeling of a protein with a molecular weight of 94,000 could be abolished by addition of unlabeled dexamethasone, indicating that the [^{3}H]dexamethasone binding is specific.

mals, suggested that the alteration in the binding kinetics of the GRc elicited by TCDD was not due to the hypoorexic effect of this chemical.

A dose-response study using TCDD doses ranging from 0.1 to 25 μg/kg demonstrated that TCDD caused a dose-dependent decrease in hepatic cytosolic [^{3}H]dexamethasone binding capacity between 1 and 10 μg/kg, with no significant effect on the equilibrium dissociation constant of the GRc (control, 0.51 ± 0.01; TCDD, 0.52 ± 0.02 nM) in sham-operated intact rats (Fig. 5A). The TCDD dose range that reduced the maximum number of hepatic GRc binding sites by 50% (ED_{50}) in the intact rats was between 3 and 5 μg/kg. Next, ADX animals were used to test whether the response to TCDD administration was modified by the adrenal status of the animals and to ensure that TCDD effects on the hepatic GRc were not due to changes in circulating glucocorticoids. For these studies, ADX female rats received a single administration of varying doses (0.1 ng to 5 μg per kg) of TCDD by gavage and were analyzed 10 days later. As stated in Materials and Methods, adrenalectomy increased the sensitivity of CD-1 rats to TCDD such that doses greater than 5 μg/kg resulted in 50 to 75% mortality. Therefore, due to this increased sensitivity of ADX rats to TCDD, the doses used to generate the dose-response curves for the ADX animals were lower than those used for the intact rats. Adrenalectomy alone greatly increased the hepatic cytosolic [^{3}H] dexamethasone binding capacity (682 ± 80 fmol/mg, n = 8) compared with the adrenal-intact controls, which was probably due to decreased levels of circulating glucocorticoid (Fig. 5A). The dose-response study of TCDD effects on the GRc of ADX animals indicated that ADX rats were much more sensitive than intact animals. A significant decrease in the GRc binding capacity of ADX rats, to 80% of corn oil-treated control values, occurred at TCDD doses as low as 0.0001 μg/kg. Although a second decrease in the hepatic GRc binding capacity of ADX rats to approximately 60% of control values was found at higher TCDD doses, the exact significance of this effect cannot be evaluated due to the toxic effects of TCDD. Although the dose-response curves for the ADX and intact animals are not parallel and a direct comparison of sensitivity cannot be made, our data do indicate that the GRc response in the ADX animals occurs at TCDD doses that are 10,000 times lower than those needed for a response in intact animals.

A monoclonal antibody specific for the rat hepatic GRc (27, 28) was used as a probe to compare the amount of GRc present in the sham-operated and ADX animals, treated with either vehicle or TCDD (sham-operated, 25 μg/kg; ADX, 5 μg/kg). These TCDD doses were chosen because they cause approximately a 50% decrease of [^{3}H]dexamethasone binding to the GRc, when compared with controls. As shown in Fig. 6A, immunoprecipitation of the GRc from crude cytosol, followed by Western blot analysis of GRc from vehicle-treated and TCDD-treated, sham-operated rats, revealed similar amounts of immunoreactive M_r 94,000 receptor. However, the same analysis of cytosol from corn oil- and TCDD-treated ADX rats did suggest that the mass of immunoreactive GRc protein in

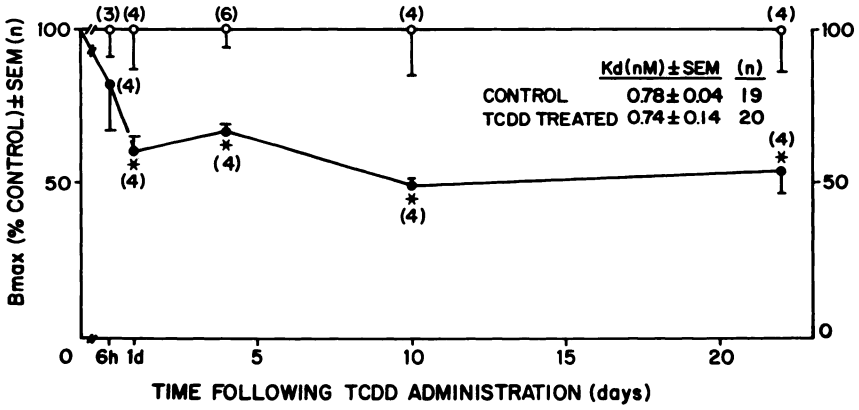
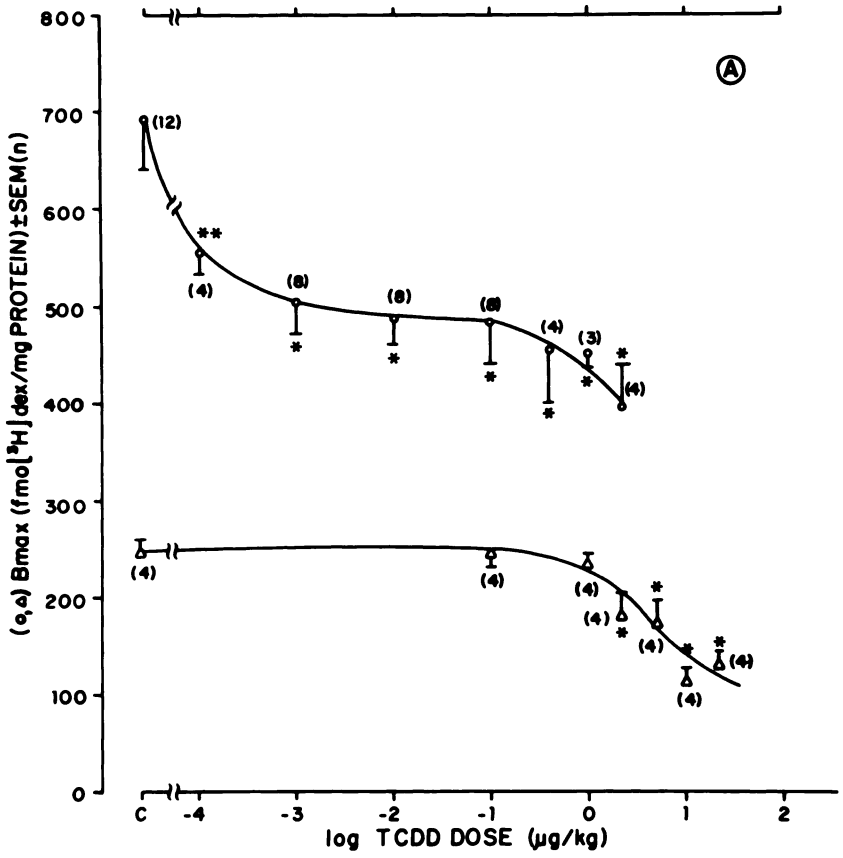


Fig. 4. Time course of the changes in the B_{max} values of the hepatic GRc following a single administration of TCDD by gavage to intact female rats. The maximum binding capacities (B_{max}) of the hepatic GRc from the TCDD-treated (25 μg/kg) rats (●) were determined using Scatchard analysis and were expressed as the percentage of B_{max} of controls (○) at each time point. The control B_{max} values were 320 ± 27, 411 ± 54, 369 ± 25, 391 ± 60, and 289 ± 41 fmol of [^{3}H] dexamethasone displaced/mg of cytosolic protein at 6 hr and 1, 4, 10, and 22 days after TCDD administration, respectively. Each data point is the mean ± standard error; (*n*), number of animals analyzed. *Significant difference ($p < 0.05$) compared with corn oil controls.



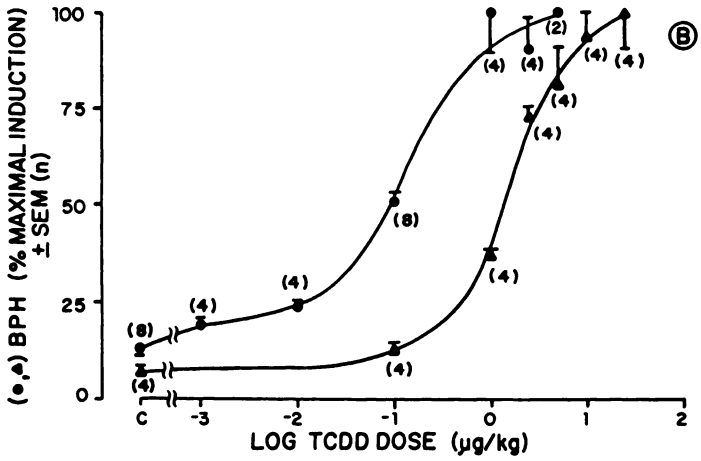


Fig. 5. Comparison of the B_{max} values of the hepatic GRc (A) and microsomal BPH (B). Maximal BPH activities (pmol/min/mg of protein) induced by TCDD were, for intact rats, 1899 ± 179 and, for ADX rats, 472 ± 40. Activities 10 days after treatment of intact (▲) or ADX (●) female rats with a single treatment of different doses of TCDD are shown. Control BPH activities (pmol/min/mg of protein) were for intact animals, 143 ± 16 and, for ADX animals, 62 ± 6. *Significant differences ($p <$ 0.05) compared with corn oil controls.

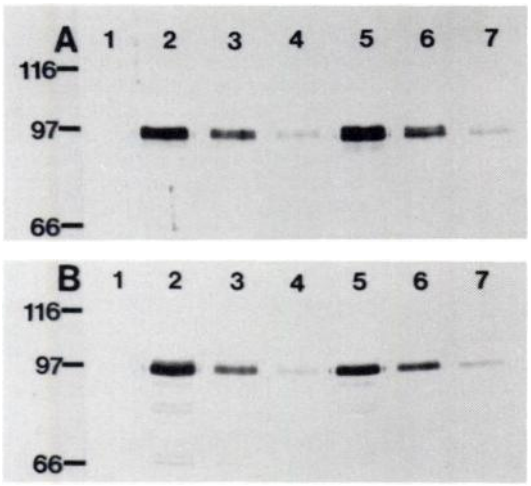


Fig. 6. Western blot analysis of hepatic GRc from vehicle or TCDD-treated, sham-operated and ADX rats. Pooled rat hepatic cytosol from corn oil- (*lanes 1–4*) or TCDD-treated (*lanes 5–7*) sham-operated (A) or ADX (B) rats was immunoprecipitated with immune mouse IgG (*lane 1*) or with BuGR2 anti-GRc monoclonal antibody (*lanes 2–7*) and was absorbed to Protein A-Sepharose, as described in Materials and Methods. Aliquots of immunoabsorbed cytosol applied to each lane of a 7% SDS-polyacrylamide gel are as follows: *lanes 1*, *2*, and *5* (400 μl); *lanes 3* and *6* (300 μl); and *lanes 4* and *7* (100 μl). Western blot analysis was performed as described in Materials and Methods.

TABLE 1

Comparison of the amount of GRc protein in cytosol from vehicle- or TCDD-treated, sham-operated and ADX rats by densitometric scanning of Western blots

The blot presented in Fig. 6 was scanned with a soft laser densitometer (Bio-Med Instruments) and peaks were excised and weighed. Values obtained from vehicle-treated samples were given an arbitrary value of 100 densitometric units. Values obtained from TCDD-treated samples were expressed relative to the vehicle-treated value. The ADX values are not comparable to the corresponding sham-operated values, because sham-operated and ADX samples (with and without TCDD) were analyzed on separate gels.

Treatment	GRc protein		
	400[a]	300	100
	arbitrary densitometric units		
Sham/corn oil	100	52.0	15.8
Sham/TCDD	115.8	56.2	14.4
ADX/corn oil	100	45.3	11.0
ADX/TCDD	71.1	40.8	9.4

ka Microliters of cytosol.

cytosol from TCDD-treated rats was slightly lower (10–30%) than that detected in vehicle-treated rats (Fig. 6B). Data obtained from soft laser scanning densitometry of the blots in Fig. 6, A and B, are summarized in Table 1. In these studies, the concentration of the antibody used to detect the rat GRc would deplete greater than 95% of the receptor labeled with [^{3}H]triamcinolone acetonide (see Materials and Methods). However, it is possible that immunoreactive saturation may still occur at some of the protein concentrations used in our assay, which would interfere with accurate quantitation of the GRc. Therefore, our Western blot data must be considered with caution until further studies can be done to ensure quantitative detection of GRc for each experimental group. Assuming that immunoreactive saturation did not occur, our data suggest that decreases in the maximum number of cytosolic [^{3}H]dexamethasone binding sites (50% reduction versus controls) are not accompanied by comparable or consistent decreases in the amount of immunoreactive GRc protein.

In order to determine whether TCDD influenced other functions of the GRc in addition to steroid binding, TCDD effects on nuclear uptake were investigated. The nuclear uptake of the receptor complex was studied using various combinations of nuclei and [^{3}H]dexamethasone-treated cytosols prepared from livers of both corn oil- and TCDD-treated intact rats (Table 2). Nuclear uptake of transformed GRc complexes resulting from combining liver nuclei ($TCDD_n$) and cytosol ($TCDD_c$) of TCDD-treated rats was 40–50% lower than that found for control nuclei ($control_n$) and cytosol ($control_c$). The reduced nuclear uptake obtained as a result of TCDD treatment was expected because of our observations that demonstrated that TCDD treatment decreased the maximum hepatic [^{3}H]dexamethasone binding capacity by 40 to 50%. However, our data indicated that the hepatic GRc that remained in TCDD-treated livers were capable of nuclear uptake. In addition, the data suggested that the nuclear uptake capacity for the steroid-GRc complex is increased by TCDD treatment, in that nuclei from TCDD-treated rat livers consistently bound more steroid-GRc than nuclei from livers of control animals. For example, when [^{3}H]dexamethasone-treated liver cytosol from control rats ($control_c$) was incubated with nuclei obtained from TCDD-treated animals ($TCDD_n$) 6 days after a single TCDD administration, the amount of control steroid-GRc complex bound to TCDD nuclei was approximately 60% greater than that observed when the same cytosol was incubated with control nuclei ($control_n$; Table 2). Also, nuclear uptake capacity of steroid-GRc complexes of cytosol from TCDD-treated livers ($TCDD_c$) was 40% greater when nuclei from TCDD-treated rats ($TCDD_n$) were used, compared with control nuclei. These data indicate that nuclear uptake of steroid-GRc complexes appeared not to be impaired by TCDD treatment; however, TCDD treatment modified liver nuclei, which resulted in an increased capacity for nuclear uptake of steroid-GRc complexes.

Past studies have demonstrated that TCDD treatment produces multiple alterations in liver biochemistry. To better understand the relationship of the GRc modification to other TCDD-mediated enzyme and receptor changes, we have compared the GRc steroid binding changes with two established biochemical markers of TCDD exposure, the induction of hepatic microsomal BPH (Fig. 5B) and the decrease in plasma membrane EGF receptor binding (Table 3). Analysis of BPH induction in the sham-operated animals (Fig. 5B) revealed that the TCDD dose that induced BPH activity by 50% (ED_{50}) was approximately 1 μg/kg, or within the ED_{50} range of the TCDD-mediated decrease in GRc B_{max} concentrations (Fig. 5A) in these animals. TCDD treatment also induced BPH activities

TABLE 2

Effect of TCDD on the nuclear uptake binding of transformed steroid-GRc complex

Intact female rats were treated with 25 μg/kg TCDD or corn oil vehicle by gavage and were sacrificed at 1 day or 6 days after treatment. For each experiment, cytosol and nuclei were prepared from livers pooled from four rats/treatment group. The data above represent the mean of duplicate assays.

Nuclei + cytosol	Specific nuclear binding	
	1 day	6 day
	dpm/μg of DNA	
$TCDD_n$ + $TCDD_c$	16.8	16.2
$Control_n$ + $control_c$	27.5	33.0
$TCDD_n$ + $control_c$	35.2	82.7
$Control_n$ + $TCDD_c$	13.5	9.7

TABLE 3

Measurements of hepatic EGF receptor

Animals received a single administration of various doses of TCDD or corn oil vehicle, 10 days before the experiment. Plasma membranes were isolated from pooled livers of four animals/treatment group. Data are expressed as the mean of duplicate determinations.

	EGF receptor binding levels	
	fmol/mg	*% of control*
Sham-operated		
Corn oil vehicle	2117	100
TCDD (μg/kg)		
1.0	1821	86
2.5	1507	71
5.0	1282	61
10.0	941	44
25.0	767	36
10–13 days after ADX		
Corn oil vehicle	2031	100
TCDD (μg/kg)		
0.001	1979	97
0.01	2571	127
0.10	2062	102
0.50	2165	107
1.0	1785	88
2.5	1049	52
5.0	1148	57

in the ADX animals; however, ADX animals exhibited increased sensitivity (ED_{50} = 0.1 μg/kg). Consistent with the previous observations (5, 6) that TCDD administration decreases hepatic plasma membrane ^{125}I-EGF receptor binding, we demonstrated that both intact and ADX animals display similar, although not completely identical, dose-dependent modifications of the EGF receptor (ED_{50} of approximately 2.5–5.0 μg/kg) (Table 3). In summary, although the dose-response curves for the GRc binding were not parallel, these experiments indicate that adrenalectomy increases the TCDD response of ADX rats for liver BPH induction by 10-fold and for the decrease in [^{3}H]dexamethasone binding by at least 10,000-fold, when compared with those of the intact controls. In contrast, the TCDD-mediated decrease in ^{125}I-EGF receptor binding was not significantly different between ADX and intact animals (Table 3). These data suggest that the adrenal status modifies specific hepatic biochemical responses to TCDD.

Discussion

The effects of TCDD on rat liver GRc were investigated and compared with other TCDD-induced hepatic biochemical responses in sham-operated and ADX adult female Sprague-Dawley rats. In our study, equilibrium binding analysis, sucrose gradient sedimentation, and affinity labeling experiments clearly demonstrated that TCDD significantly reduced [^{3}H] dexamethasone binding to the hepatic GRc, due to a decrease in the number of binding sites (B_{max}) of the receptor, with little effect on the apparent equilibrium dissociation constant (K_d). Although the Western blot data must be considered with caution because of possible immunoreactive saturation (see Results), the decrease in the maximum number of GRc binding sites did not appear to be accompanied by a consistent or equivalent reduction in the level of immunodetectable GRc protein in ADX or intact animals. These data suggest that TCDD administration results in a modification of the ability of hepatic GRc to bind glucocorticoids without altering the actual levels of the receptor protein in the liver. In addition, the Western blot analysis demonstrated that the levels of immunodetectable hepatic GRc found in ADX animals were only slightly higher than the levels found in the sham-operated control rats, even though the GRc binding capacities were twice those measured in the sham-operated controls. These data may be explained by the occupation of hepatic GRc by endogenous steroids present in the sham-operated controls (see Results). Unfortunately, it is impossible to evaluate occupancy of the GRc because there is not an adequate exchange assay available.

Although hepatic steroid binding to the GRc was reduced, TCDD treatment did not appear to interfere with the nuclear uptake of the steroid-GRc complex. However, TCDD treatment did result in an increased capacity for nuclear uptake of the steroid-GRc complex. It is of interest that a number of hepatocarcinogens, in contrast to TCDD, reduce the nuclear uptake capacity for the hepatic steroid-GRc complex (15). Although the significance of modulating the nuclear uptake of the glucocorticoids is not known, these changes may alter the response of the liver to glucocorticoids. It has been proposed that the reduction of glucocorticoid binding to liver nuclei, after carcinogen treatment, is responsible for the inhibition of hydrocortisone induction of liver tyrosine aminotransferase and tryptophan pyrolase (15). Although we have not investigated tyrosine aminotransferase induction in our strain of female rats, one study has shown that TCDD (50 μg/kg) administration did not significantly alter dexamethasone-stimulated tyrosine aminotransferase activities in the adult male rat (10). Another recent study has demonstrated that the effect of TCDD on the GRc is not limited to the liver (37). Max and Silbergeld (37) have shown that toxic doses of TCDD decreased the GRc binding capacities in rat skeletal muscle, in association with the TCDD-mediated wasting syndrome. The physiological significance of TCDD effects on tissue GRc binding and nuclear uptake is not yet clear.

Although the mechanism by which TCDD influences GRc binding is not yet known, there are several factors that have been shown to influence the function of the GRc without modifying actual levels of receptor protein. The GRc undergoes two types of covalent modification, phosphorylation of serine residues and reduction of sulfur groups, that affect both the steroid- and DNA-binding activities (38, 39). Grippo *et al.* (39) have demonstrated that reduced sulfhydryl groups are required for the GRc to bind steroids and for the transformed receptor complex to bind DNA. Housley and Pratt (38) have demonstrated that phosphorylation of the GRc by cAMP-dependent protein kinase increased steroid binding. It is of interest that significant increases in the activities of cAMP-dependent protein kinase and protein kinase C have been demonstrated in isolated rat hepatic plasma membranes 10 to 20 days after TCDD treatment (40). These kinases are involved in the regulation of peptide and steroid receptor function and, therefore, may represent one mechanism by which TCDD modulates both the EGF and glucocorticoid receptor pathways. Modification of the cellular sulfhydryl-reducing apparatus and/or protein kinase activities by TCDD would profoundly influence the functioning of not only the GRc, but also other steroid receptor pathways, without modifying receptor protein levels. Evidence that TCDD may have a general effect and modify other steroid hormone receptor systems was provided by a recent study (41), which demonstrated that TCDD treatment significantly decreased hepatic and uterine estrogen receptor binding in female

Long-Evans rats. In addition, a number of inducers of hepatic mixed function oxidases have been shown to decrease cytosolic estrogen binding in ovariectomized rats (42). The effect of these compounds on steroid receptors may alter normal physiological functioning of the liver. An alteration in hormone response may in turn be important to the tissue-specific actions of these carcinogens and toxins.

Our TCDD dose-response studies compared the GRc steroid binding changes with two other biochemical markers of TCDD exposure, BPH induction and the reduction in hepatic EGF receptor binding. Consistent with previous reports, TCDD administration significantly increased hepatic microsomal BPH activities and decreased hepatic plasma membrane EGF receptor levels in the intact rat, in a dose-dependent manner (5–8). In the intact animal, GRc binding response to TCDD occurred within the same dose range (3–5 μg/kg) as that for BPH induction and the decrease in EGF receptor concentration. In ADX rats, even though the dose-response curves were not parallel, our data do demonstrate that rats were much more sensitive to TCDD, in that a significant decrease in hepatic GRc binding occurred at doses that were 10,000 times lower than those required for a response in intact animals. In addition, increased sensitivity of microsomal BPH induction (10-fold) and no detectable alteration in sensitivity of EGF receptor binding to TCDD were observed in the ADX, compared with intact animals. Our data support the hypothesis that the action of TCDD is dramatically modified by the adrenal status of the animal; however, the mechanism remains unclear. The effect is not likely to be related to changes in TCDD metabolism and/or distribution, inasmuch as TCDD is very slowly metabolized and our preliminary results (not shown) indicate that adrenalectomy has no effect on TCDD concentrations in liver.

The *Ah* locus has been shown to be necessary to elicit many of the TCDD-induced biochemical changes, but whether the *Ah* locus is also involved in the effects of TCDD on the GRc is not yet clear. TCDD promotion of skin cancer in the hairless mouse indicates that at least two genetic loci are required, the *Ah* and the *hr* loci (8). Studies to determine more precisely the role of the Ah receptor system in mediating the GRc response are in progress, using congenic mice with and without a mutation of the *Ah* receptor locus. Taken together, our results have characterized the effects of TCDD on the GRc and have revealed that adrenalectomy sensitizes rats to the TCDD-mediated decreases in binding of the GRc to dexamethasone.

References

1. McConnell, E. E., J. A. Moore, J. K. Haseman, and M. W. Harris. The comparative toxicity of chlorinated dibenzo-*p*-dioxin in mice and guinea pigs. *Toxicol. Appl. Pharmacol.* **44**:335–356 (1978).
2. Kimbrough, R. D. *Halogenated Biphenyl, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products.* Elsevier/North Holland, New York (1980).
3. Schwetz, B. S., J. M. Norris, G. L. Sparschv, V. K. Rowe, P. J. Gehring, J. L. Emerson, and G. G. Gerbig. Toxicology of chlorinated dibenzo-*p*-dioxins. *Environ. Health Perspect.* **5**:87–99 (1973).
4. Kociba, R. J., D. G. Keyes, J. E. Beyer, R. J. Carreon, C. E. Wade, D. A. Dittenber, R. P. Kalnins, L. Frauson, C. N. Park, S. D. Barnard, R. A. Hummel, and C. G. Humiston. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. *Toxicol. Appl. Pharmacol.* **46**:279–303 (1978).
5. Pitot, H. C., and A. E. Surica. The stages of initiation and promotion in hepatocarcinogenesis. *Biochim. Biophys. Acta* **605**:191–215 (1980).
6. Makhukar, B. V., D. W. Brewster, and F. Matsumura. Effects of *in vivo* administered 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on receptor binding of epidermal growth factor in the hepatic plasma membrane of rat, guinea pig, mouse and hamster. *Proc. Natl. Acad. Sci. USA* **81**:7407–7411 (1984).
7. Poland, A., E. Glover, and A. S. Kende. Stereospecific high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. *J. Biol. Chem.* **251**:4936–4946 (1976).
8. Poland, A., and J. C. Knutson. 2,3,7,8-Tetrachlorodibenzo- and related halogenated aromatic hydrocarbons: examination of the mechanisms of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22**:517–754 (1982).
9. Vos, J. G., J. A. Moore, and J. G. Zinkl. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the immune system of laboratory animals. *Environ. Health Perspect.* **5**:149–162 (1973).
10. Neal, R. A., P. W. Beatty, and T. A. Gasiewicz. Studies on the mechanisms of toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Ann. N. Y. Acad. Sci.* **320**:204–213 (1979).
11. Peterson, R. E., C. L. Potter, and R. W. Moore. The wasting and hormonal alterations in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity, in *Public Health Risks of the Dioxins* (W. W. Lowrance, ed.). William Kaufmann, Los Altos, CA, 315–349 (1984).
12. Walters, M. R. Steroid hormone receptors and the nucleus. *Endocrine Rev.* **6**:512–543 (1985).
13. Grody, W. W., W. T. Schrader, and B. W. O'Malley. Activation transformation and subunit structure of steroid hormone receptors. *Endocrine Rev.* **3**:141–163 (1982).
14. Hunter, W. M., and F. C. Greenwood. Preparation of iodine-131 human growth hormone of high specific activity. *Nature* (*Lond.*) **194**:495–496 (1962).
15. Kensler, T. W., W. F. Busby, Jr., N. E. Davidson, and G. N. Wogan. Effect of hepatocarcinogens on the binding of glucocorticoid-receptor complex in rat liver nuclei. *Cancer Res.* **36**:4647–4651 (1976).
16. Higgins, S. J., G. G. Rousseau, J. D. Baxter, and G. M. Tomkins. Early events in glucocorticoid action. *J. Biol. Chem.* **248**:5866–5872 (1973).
17. Neville, D. M. Isolation of an organ specific protein antigen from cell-surface membrane of rat liver. *Biochim. Biophys. Acta* **154**:540–552 (1968).
18. Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1979).
19. Rosner, W., and S. T. Polimeni. An exchange assay for the cytoplasmic glucocorticoid receptor in the liver of the rat. *Steroids* **31**:427–438 (1978).
20. Beato, M., and P. Feigelson. Glucocorticoid-binding proteins of rat liver cytosol. I. Separation and identification of the binding proteins. *J. Biol. Chem.* **247**:7890–7896 (1972).
21. Scatchard, G. The attraction of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
22. Downs, T. R., and N. W. Wilfinger. Fluorometric quantification of DNA in cells and tissue. *Anal. Biochem.* **131**:538–547 (1983).
23. Eastman-Reks, S. B., C. E. Reker, and M. V. Vedeckis. Structure and subunit dissociation of the mouse glucocorticoid receptor: rapid analysis using vertical tube rotor sucrose gradients. *Arch. Biochem. Biophys.* **230**:1–7 (1984).
24. Griffith, O. M. Density gradient separations in ventricle tube, fixed angle and swinging bucket rotors: a comparative study. *Applications Data DS-602.* Beckman Instruments Inc. Spinco Division, CA (1984).
25. Martin, R. G., and B. N. Ames. A method of determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236**:1372–1379 (1961).
26. Eisen, H. J., R. E. Schleenbaker, and S. S. Simons, Jr. Affinity labeling of the rat liver glucocorticoid receptor with dexamethasone 21-mesylate. *J. Biol. Chem.* **256**:12920–12925 (1981).
27. Gatmetchu, B., and R. W. Harrison. Characterization of a monoclonal antibody to the rat liver glucocorticoid receptor. *Endocrinology* **114**:274–279 (1984).
28. Eisen, L. P., M. E. Reichman, E. B. Thompson, B. Gametchu, R. W. Harrison, and H. J. Eisen. Monoclonal antibody to the rat glucocorticoid receptor: relationship between the immunoreactive and DNA-binding domain. *J. Biol. Chem.* **261**:11805–11810 (1985).
29. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* (*Lond.*) **227**:680–685 (1970).
30. Sanchez, E. R., D. O. Toft, M. J. Schlessinger, and W. B. Pratt. Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. *J. Biol. Chem.* **260**:12398–12401 (1985).
31. Housley, P. R., E. R. Sanchez, H. M. Westphal, M. Beato, and W. B. Pratt. The molybdate-stabilized L-cell glucocorticoid receptor isolated by affinity chromatography or with a monoclonal antibody is associated with a 90–92-kDa nonsteroid-binding phosphoprotein. *J. Biol. Chem.* **260**:13810–13817 (1985).
32. Carpenter, G., and S. Cohen. ^{125}I-labelled human epidermal growth factor: binding, internalization and degradation in human fibroblasts. *J. Cell. Biol.* **71**:159–171 (1976).
33. Nebert, D. W., and H. V. Gelboin. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. *J. Biol. Chem.* **243**:6242–6249 (1968).
34. Deleted in proof.
35. Snedecor, G. W., and W. G. Cochran. *Statistical Methods*, 7th Ed. Iowa State University Press, Ames, IA (1980).
36. Weiland, G. A., and P. B. Molinoff. Quantitative analysis of drug receptor interactions. I. Determination of kinetic and equilibrium properties. *Life Sci.* **29**:313–330 (1981).
37. Max, S. R., and E. K. Silbergeld. Skeletal muscle glucocorticoid receptor and glutamine synthetase activity in the wasting syndrome in rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmacol.* **87**:523–527 (1987).

38. Housley, P. R., and W. B. Pratt. Direct demonstration of glucocorticoid receptor phosphorylation by intact L-cells. *J. Biol. Chem.* **258**:4630–4635 (1983).
39. Grippo, J. F., A. Holmgren, and W. B. Pratt. Proof that the endogenous, heat-stable glucocorticoid receptor-activating factor is thioredoxin. *J. Biol. Chem.* **260**:93–97 (1985).
40. Nelson, K., A. Vickers, G. Sunahara, and G. Lucier. Receptor and DNA ploidy changes during promotion of rat liver carcinogenesis, in *Tumor Promoters: Biological Approaches for Mechanistic Studies and Assay Systems*. (R. Langenbach and C. Barrett, eds.). Raven Press, New York, 407–416 (1987).
41. Romkes, M., J. Piskorska-Pliszczyneka, and S. Safe. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on hepatic and uterine estrogen receptor levels in rats. *Toxicol. Appl. Pharmacol.* **87**:306–314 (1987).
42. Duvivier, J., J. Van Cantfort, and J. E. Gielen. The influence of five monooxygenase inducers on liver cytosol estradiol receptor levels in the ovariectomized adult rat. *Biochem. Biophys. Res. Commun.* **99**:252–258 (1981).

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