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NAD(P)H:QUINONE OXIDOREDUCTASE (DT-DIAPHORASE) IN CHICK EMBRYO LIVER

COMPARISON TO ACTIVITY IN RAT AND GUINEA PIG LIVER AND DIFFERENCES IN CO-INDUCTION WITH 7-ETHOXYRESORUFIN DEETHYLASE BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN

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Abstract—NAD(P)H:quinone oxidoreductase (EC 1.6.99.2; DT-diaphorase) was present in the liver of 18- and 19-day-old chick embryos as assayed both by reduction of resorufin and by the more traditional assay, reduction of 2,6-dichlorophenolindophenol (DCPIP). Both reductions had the classic characteristics of DT-diaphorase: they were equally supported by NADPH and NADH and almost entirely inhibited by dicumarol. Chick embryo liver DT-diaphorase was entirely cytosolic. It was undetectable in the microsomal and mitochondrial fractions. Chick embryo liver cytosol and mitochondrial fractions contained an enzyme oxidizer of resorufin but not of DCPIP. The K_m for NADPH for resorufin reductase was an order of magnitude higher in chick embryo than in rat or guinea pig cytosol (1 mM vs 0.1 mM). Resorufin reductase activity was higher for chick embryo than for rat or guinea pig cytosols: V_{max} (nmol resorufin reduced per mg cytosolic protein per min ± SEM) 355 ± 28 for chick embryo, 159 ± 10 for guinea pig and 68 ± 28 for rat. The V_{max} for DCPIP reduction was also twice as high in chick embryo as rat liver cytosol. In the chick embryo, 7 days after treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at 6.4 μg/kg egg (1 nmol/egg) mortality was increased 2.4-fold, hepatic DT-diaphorase 1.3-fold, and 7-ethoxyresorufin deethylase (7-EROD) 72-fold over control levels. At 32 μg/kg, mortality was increased 4.2-fold, DT-diaphorase 2.3-fold and 7-EROD 100-fold. In the guinea pig, 5 days after treatment with TCDD at 10 μg/kg, TCDD toxicity was also evident (loss of body weight and thymus weight); there was no change in DT-diaphorase as measured by resorufin reduction, confirming by a different assay the observation of Beatty and Neal (*Biochem Pharmacol* **27**: 505–510, 1978) that TCDD does not induce DT-diaphorase in guinea pig liver, and 7-EROD was increased 8-fold. In contrast, in the rat, 7 days after exposure to TCDD at 10 μg/kg, there was no evidence of toxicity, DT-diaphorase was increased close to 7-fold and 7-EROD, 100-fold. The results demonstrate that avian liver contains DT-diaphorase and show that the extent to which DT-diaphorase is part of the pleiotypic response of the liver to an *Ah* (*a*ryl *h*ydrocarbon) receptor ligand is species dependent. They also suggest that DT-diaphorase induction and TCDD toxicity may be inversely related. The possibility that DT-diaphorase protects against TCDD toxicity and participates in species differences in sensitivity to TCDD toxicity warrants further investigation.

DT-Diaphorase (NAD(P)H:quinone oxidoreductase, EC 1.6.99.2) catalyzes the two-electron reduction of quinones and quinone imines to relatively stable hydroquinones and aminophenols that are readily conjugated with glucuronate or sulfate and excreted [1–4]. In contrast, NAD(P)H cytochrome *c* reductase (EC 1.6.99.1) catalyzes the one-electron reduction of quinones and quinone imines to highly reactive semiquinone free radicals that can be toxic themselves or metabolized to other reactive oxygen species. It has been suggested that two-electron reduction by DT-diaphorase competes with the one-electron reduction and protects against cytotoxicity from quinones or chemicals metabolized to quinones [3–8].

DT-Diaphorase is coordinately induced with cytochrome P-448‡ mediated mixed-function oxidases such as aryl hydrocarbon hydroxylase (AHH†) and 7-ethoxyresorufin deethylase (7-EROD) by *Ah* receptor ligands like 3-methylcholanthrene (3-MC), benzo[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in livers of mouse, rat and hamster [2, 9–11] but not of guinea pig [10]. The mechanisms involved in TCDD toxicity, in protection against toxicity, and in species differences in sensitivity to TCDD toxicity are not understood. As DT-diaphorase offers potential protection against chemical toxicity and is not induced in liver of the guinea pig, the species most sensitive to TCDD toxicity, we asked whether DT-diaphorase was inducible in liver of the chick embryo, another highly sensitive species [12–

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† Abbreviations: *Ah*, *a*ryl *h*ydrocarbon; AHH, aryl hydrocarbon hydroxylase; BSA, bovine serum albumin; DCPIP, 2,6-dichlorophenolindophenol; 7-EROD, 7-ethoxyresorufin deethylase; FAD, flavin adenine dinucleotide; 3-MC, 3-methylcholanthrene; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

‡ P-448 refers to the cytochrome P-450 isoforms induced by *Ah* receptor agonists. The chick liver isoforms in this class analogous to the rat and rabbit liver isoforms that have been sequenced and assigned to IA gene family are not fully characterized but have similar induction responses and molecular weights by SDS–PAGE.

14].* We were further prompted in this investigation by the fact that the enzyme in chick liver had not been characterized.

An earlier study failed to find the enzyme in pigeon mitochondria [15] leading to the impression that DT-diaphorase was absent from avian liver. We found, however, substantial DT-diaphorase activity in chick embryo liver cytosol assayed by two independent methods, reduction of resorufin, a recently reported assay for DT-diaphorase [2], and of 2,6-dichlorophenolindophenol (DCPIP), a well-established assay [1]. The DT-diaphorase activity of chick embryo cytosol was in fact greater than that of rat or guinea pig liver cytosol. However, in liver of chick embryo like in guinea pig, TCDD effected little induction of DT-diaphorase.

This paper reports on characteristics of DT-diaphorase in chick embryo liver and compares activities in chick embryo, guinea pig and rat liver and the induction effects of TCDD on 7-EROD and DT-diaphorase in the three species. Our results demonstrate that the extent to which TCDD coinduces DT-diaphorase and mixed-function oxidase activity differs among species. Furthermore, the findings are compatible with an inverse relationship between DT-diaphorase inducibility and species sensitivity to TCDD toxicity.

MATERIALS AND METHODS

Materials. TCDD was a gift of Dr Alan Poland, McArdle Institute for Cancer Research, Madison, WI. 7-Hydroxyphenoxazone (resorufin) and 7-ethoxyphenoxazone (7-ethoxyresorufin) were obtained from the Pierce Chemical Co. (Rockford, IL), DCPIP, NADPH, NADH, Tris-base, Tris–HCl, potassium phosphate (monobasic and dibasic) and 3,3′-methylene-bis-(4-hydroxycoumarin) (dicumarol) from the Sigma Chemical Co. (St. Louis, MO).

Animals, treatment and tissue preparation. Chick embryos (White Leghorn strain), Shamrock Farms, North Brunswick, NJ, were incubated at 37° and 70% relative humidity. TCDD was injected into 11- to 12-day-old fertilized eggs through a hole in the shell into the fluids surrounding the embryo at 1 or 5 nmol/egg (6.4 or 32.2 μg/kg egg) in 0.01 mL of dioxane. Controls received solvent alone. After incubation for 7 days, the livers were perfused *in situ* through the heart with cold 0.1 M potassium phosphate buffer, pH 7.4, removed, weighed, pooled (4–6 per treatment group), homogenized in 0.1 M cold potassium phosphate buffer, pH 7.4 (25%, w/v), and centrifuged for 10 min at 9000 *g*. The 9000 *g* pellet, containing the mitochondria, was used as an enzyme source in some experiments. The supernatant fraction was centrifuged at 105,000 *g* for 1 hr to make microsomes (105,000 *g* pellet) and cytosol (supernatant) [14]. Tissue fractions were assayed directly or frozen at −80°. Protein was measured by the method of Lowry *et al.* [16].

Male Hartley guinea pigs, 300–350 g, and male Sprague–Dawley rats, 150–200 *g*, were injected i.p. with TCDD at 10 μg/kg in corn oil or an equivalent volume of corn oil alone (controls). After 5–7 days, animals were weighed and killed after exposure to light CO_2 vapors by a blow to the head. Subcellular fractions were prepared from individual livers, as described above for chick embryo.

DT-Diaphorase. DT-Diaphorase was assayed (a) fluorometrically by the reduction of resorufin [2] and (b) spectrophotometrically by the reduction of DCPIP [1]. For the former, unless specified otherwise, reaction mixtures contained resorufin, 1.25 to 20 μM (dissolved in dimethyl sulfoxide and diluted 100-fold or more in Tris–HCl), cytosol or other subcellular fractions and Tris–HCl, 0.05 M, pH 7.5, in a volume of 0.24 mL, in 6 × 50 mm tubes. Reactions were started with NADPH at concentrations determined to be non-limiting (see Results). The decrease in resorufin fluorescence at room temperature was monitored in a Perkin–Elmer MPF-3 fluorescence spectrophotometer (excitation λ, 522 nm; emission λ, 586 nm) for at least 100 sec (when resorufin reduction was usually 90% complete). Substrate utilization was measured by reference to resorufin standards.

Exogenous FAD, a cofactor for DT-diaphorase, is needed to measure activity of pure enzyme and in cytosol of mouse liver [17], but not for rat, hamster or guinea pig cytosol. FAD at 0.5 to 50 μM had no significant effect on resorufin reduction in cytosol of control or TCDD-treated livers of any of the species tested and was inhibitory at 500 μM. Accordingly, it was not included in these experiments.

To measure DCPIP reduction, unless otherwise indicated, reaction mixtures contained NADPH, 0.5 mM, bovine serum albumin (BSA), 0.07% (w/v), DCPIP at 60 μM, cytosol or other subcellular fractions and Tris–HCl, 0.05 M, pH 7.5. Reactions were started with DCPIP and followed for at least 100 sec. The sample cuvette contained the full reaction mixture and the reference cuvette, the same mixture without DCPIP. The rate of DCPIP reduction at room temperature was measured by the decrease in absorbance at 600 nm using an extinction coefficient of 21/mM/cm. For both methods, initial rates were used to measure DT-diaphorase activity. Experiments were also performed with dicumarol added at the start of the reaction or after the substrate had been reduced. K_m and V_{max} values were determined from double-reciprocal plots.

7-Ethoxyresorufin deethylase (7-EROD) activity. 7-EROD was measured in microsomes from livers from which cytosol was assayed for DT-diaphorase as reported [18] except that dicumarol was added to inhibit any resorufin reductase that might be present [19]. Reaction mixtures (in 6 × 50 mm tubes) contained NADPH, 1 mM; dicumarol, 10 μM; microsomes equivalent to 0.5 mg wet weight of liver; and

* The chicken, like the guinea pig, is highly sensitive to TCDD toxicity [12]. The chick embryo at 18–20 days (close to hatching) exhibits the same indicia of TCDD toxicity as does the adult chicken [13] (thymic involution, edema, increased mortality). Moreover, its cytochromes P-450 and related drug-metabolizing enzyme activities are highly inducible by both *Ah*-receptor ligands and inducers of the phenobarbital class, and levels are comparable to the adult chicken [12–14]. In those respects, the chick embryo contrasts markedly with the mouse and rat fetus, in which mixed-function oxidase activity and inducibility are extremely low prior to birth and undergo dramatic increases only in the neonatal period.

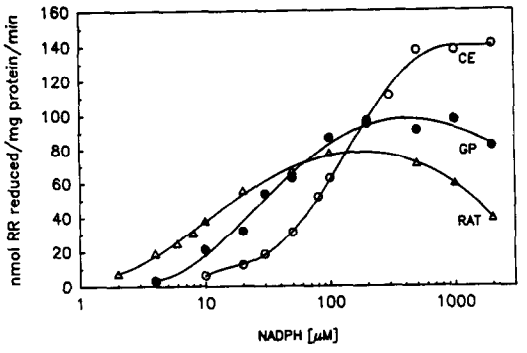

Fig. 1. NADPH-dependence of resorufin (RR) reduction by chick embryo (○), guinea pig (●) and rat (△) liver cytosols. Cytosols were prepared and assayed as described in Materials and Methods. Reaction mixtures contained cytosol (19, 30 and 27 μg protein for chick embryo, guinea pig and rat respectively); resorufin, 10 μM; and Tris–HCl, 0.05 M, pH 7.5, in a total volume of 0.22 mL. NADPH was added in 0.02 mL of the Tris–HCl, 0.05 M.

Tris–HCl, 0.05 M, pH 7.5, in a total volume of 0.24 mL. Reactions were initiated with 7-ethoxyresorufin, 2 μM final concentration, carried out for 5 min at 37° in a shaking water bath in air, and stopped with cold acetone, 0.25 mL. The product, resorufin, was measured in the same tubes following centrifugation at 3500 rpm for 10 min, at excitation and emission λ of 558 and 586 nm respectively. An excitation λ of 558 nm was used in this assay to avoid interference from fluorescence of 7-ethoxyresorufin at 522 nm [20]. Product (resorufin) formation was measured by reference to a quinine sulfate standard that had been calibrated previously against resorufin.

Statistical significance of differences in means was evaluated by Student's *t*-test.

Toxicity. Body weights were measured before and after treatment and thymus weights, after treatment, for guinea pigs and rats. The incidence of mortality was tabulated for the chick embryos and evaluated by χ^2 tests.

RESULTS

Resorufin reduction by chick embryo, rat and guinea pig cytosol: NADPH requirements. Resorufin was reduced by cytosols of chick embryo, guinea pig and, as reported [2], by rat liver. Chick embryo cytosol required more NADPH for maximal reduction rates than did cytosols of the other species (Fig. 1). The K_m values (μM) for NADPH were 201, 46 and 16 for chick embryo, guinea pig and rat cytosols respectively. At non-limiting NADPH, resorufin reductase activity was greater in chick embryo than in guinea pig or rat cytosol, e.g. for the experiment in Fig. 1, the V_{max} values (nmol resorufin reduced per mg cytosolic protein per min) were 187, 117 and 95 for chick embryo, guinea pig and rat respectively. NADPH was inhibitory at concentrations over 0.5 mM for rat, 1.0 mM for guinea pig and 5.0 mM (data not shown) for chick embryo cytosols. In subsequent experiments resorufin reduction was assayed at NADPH concentrations yielding maximal rates: 1 mM for chick embryo and 0.1 mM for guinea pig and rat cytosols.

Reaction characteristics. Resorufin reduction was a linear function of cytosol concentration to 50 μg protein per reaction mixture for chick embryo, 200 μg for rat and at least 75 μg for guinea pig. Routinely, cytosols from all three species were assayed at 19–32 μg protein per reaction mixture. At concentrations of cytosol giving the same initial rate, the time course of the reaction was linear for a shorter period in chick embryo (10–30 sec) than in rat or guinea pig cytosol (up to 60 sec). When the reaction was complete, the fluorescence remained fully suppressed for up to 20 min (the longest time examined).

Dicumarol, an inhibitor of DT-diaphorase [1], inhibited resorufin reduction by chick embryo liver cytosol when added to reaction mixtures before initiating the reaction (greater than 95% inhibition at 10 μM dicumarol). Dicumarol added after reduction occurred caused the fluorescence to return to baseline levels, indicating that resorufin was reoxidized. The initial reoxidation rate for resorufin by dicumarol in chick embryo cytosol was about one-third of the initial reduction rate and in rat and guinea pig cytosol was about one-half the rate for chick embryo cytosol. The faster reoxidation of resorufin by chick embryo cytosol suggested that enzymic oxidation occurs in chick embryo cytosol in addition to the non-enzymic oxidation by air [2]; both are unopposed when the reductase is inhibited. Greater oxidation of resorufin would explain the shorter linearity of the reduction reaction in chick embryo than in rat and guinea pig cytosol.*

For all three species resorufin reduction was maximal at about 15 μM resorufin and there was substrate inhibition at resorufin concentrations over 20 μM (Fig. 2). The substrate inhibition precluded deter-

* Related observations on resorufin reduction and reoxidation are as follows: (1) Acetone, 50% (v/v), in the reaction mixture, like dicumarol, prevented resorufin reduction when added prior to the reaction and provoked reoxidation when added after the reaction was complete. The initial reoxidation rate with acetone was the same in all three species and was the same as the reoxidation rate with dicumarol in guinea pig and rat cytosol. This result supports the hypothesis that chick embryo cytosol contains an enzymic oxidizer of resorufin (dicumarol, a specific inhibitor of the reductase would be expected to inhibit only the reductase and acetone to inhibit enzymic activity more generally). (2) The observed resorufin reduction rates are the net resultant of reduction and oxidation (both non-enzymic and enzymic). The non-enzymic oxidation would be the same in all three species. The presence of an additional enzymic oxidizer in chick cytosol would have little impact on the initial reduction rates used to compare resorufin reduction in all three species because at the beginning of the reduction all of the substrate is present in the oxidized state and there is no reduced substrate available for the oxidizer. (3) Dicumarol also prevented reduction of DCPIP when added at the beginning of the reaction and caused reoxidation of DCPIP when added after reduction was complete. The enzymic oxidizer was selective for resorufin as the rate of reoxidation of DCPIP by dicumarol did not differ for the three species. (4) Dicumarol did not affect the relative fluorescence or the excitation or emission spectra for resorufin. Acetone, 50% (v/v), increased resorufin fluorescence by 70% both with and without cytosol and before or after reduction had occurred. Acetone also shifted the emission maximum of resorufin from 586 to 592 nm.

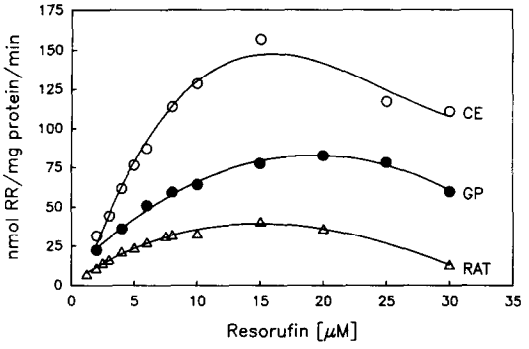

Fig. 2. Resorufin reduction as a function of resorufin concentration in chick embryo (○), guinea pig (●) and rat (△) liver cytosols. Reaction mixtures contained cytosol (23, 32 and 26 μg protein for chick embryo, guinea pig and rat respectively); resorufin, 1.25 to 30 μM; NADPH (1 mM for chick embryo; 0.1 mM for guinea pig and rat); and Tris–HCl, 0.05 M, pH 7.5, in a total volume of 0.24 mL. Assays were performed as described in Materials and Methods. In the experiments shown, the K_m values (μM) for resorufin were 22, 11 and 8, and the V_{max} values (nmol resorufin reduced per mg cytosolic protein per min) were 411, 140 and 70 for chick embryo, guinea pig and rat respectively.

Table 1. Cofactor dependence and subcellular distribution of resorufin reductase activity in chick embryo liver

Liver fraction	Cofactor	% of Control
Cytosol	NADPH	100
Cytosol	NADH	101
Cytosol	None	0
Heated cytosol	NADPH	0
Cytosol + heated cytosol	NADPH	98
9000 *g* Supernatant	NADPH	108
Microsomes	NADPH	3
9000 *g* Pellet	NADPH	0
Homogenate	NADPH	40
Cytosol + microsomes	NADPH	90
Cytosol + 9000 *g* pellet	NADPH	50

Reaction mixtures were in a total volume of 0.24 mL and contained 10 μM resorufin and subcellular liver fractions or homogenate equivalent to 0.375 mg wet weight. The concentration of NADPH or NADH, when present, was 1 mM. Resorufin reduction was measured as described in Materials and Methods. Mean resorufin reduction ± SEM in reaction mixtures containing resorufin, cytosol and NADPH (control = 100%) was 119 ± 6 nmol resorufin reduced per mg protein per min (N = 3). Where indicated, cytosol was heated at 60° for 10 min.

mining V_{max} by direct assay. Accordingly, in subsequent experiments, DT-diaphorase activity was calculated from double-reciprocal plots of measurements made at multiple substrate concentrations.

Modifications of resorufin reduction and distribution of activity in subcellular fractions of chick embryo liver: evidence that resorufin reductase in chick embryo liver is a DT-diaphorase. Resorufin reduction by chick embryo cytosol was supported by NADH or NADPH. It did not occur in the absence of reduced pyridine nucleotides (Table 1). The K_m values for NADPH and NADH were 201 and 53 μM. The NADPH- and NADH-supported reductions had essentially the same V_{max}, 187 and 160 nmol resorufin reduced per mg cytosolic protein per min, respectively (Fig. 3). Resorufin was reduced less than 5% by NADPH or cytosol alone or by cytosol heated to 60° for 10 min, demonstrating that the cytosolic reduction was enzymic. Heated cytosol added to reaction mixtures containing unheated cytosol did not affect the reduction rate, indicating that cytosol did not contain non-enzymic activators or inhibitors of the reduction.

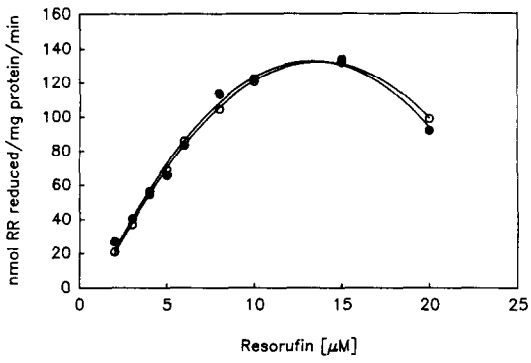

Fig. 3. Comparison of resorufin reduction using NADPH (●) or NADH (○), as cofactors. Reactions were performed as described in Materials and Methods. The same chick embryo liver cytosol (control), 23 μg protein per reaction mixture, was used for both cofactors.

The reduction rate for 9000 *g* supernatant fraction, which contains cytosol and microsomes, was the same as for cytosol alone. Microsomes and the 9000 *g* pellet, which contains mitochondria, did not reduce resorufin (Table 1) or DCPIP. The 9000 *g* pellet, in contrast, *increased* resorufin fluorescence, suggesting that it contained an oxidizer of resorufin. Resorufin reduction by the 9000 *g* pellet added to cytosol was about 50% of the rate in cytosol alone. Homogenate reduced resorufin but only at 40% of the rate in cytosol, probably because homogenate contained the 9000 *g* pellet and its oxidizing factor. The oxidizing effect of the pellet was substrate selective as it was not observed using DCPIP as a substrate. Reduction rates for DCPIP in the homogenates and cytosol were essentially the same.

Comparison of the effects of TCDD on resorufin reduction in chick embryo, guinea pig and rat liver cytosol. Figure 4 shows Lineweaver–Burk plots for resorufin reduction by liver cytosols of control and TCDD-treated chick embryo, guinea pig and rat (mean collective data), and Table 2, the mean K_m and V_{max} values for individual experiments. For controls the K_m values (μM) for resorufin were 17 ± 2, 9 ± 1 and 8 ± 1 (mean ± SEM) for chick embryo, guinea pig and rat cytosols, respectively, and the V_{max} values (nmol resorufin reduced per mg cytosolic protein per min) were: 355 ± 28, 159 ± 10 and 68 ± 28 (mean ± SEM).

In chick embryo liver, TCDD at 1 nmol/egg (6.4 μg/kg egg) did not affect the K_m for resorufin and increased the V_{max} slightly [to 1.3 times the mean control values ($P < 0.01$)]. At 5 nmol/egg (32 μg/kg), TCDD increased the K_m to 3.1 times and the V_{max} to 2.75 times control values ($P < 0.05$ for K_m and $P < 0.01$ for V_{max} for each parameter compared to both control and the lower dose of TCDD). (The higher K_m in the high dose TCDD-treated embryos suggests that TCDD increased a DT-diaphorase isozyme different from that in control livers or the same

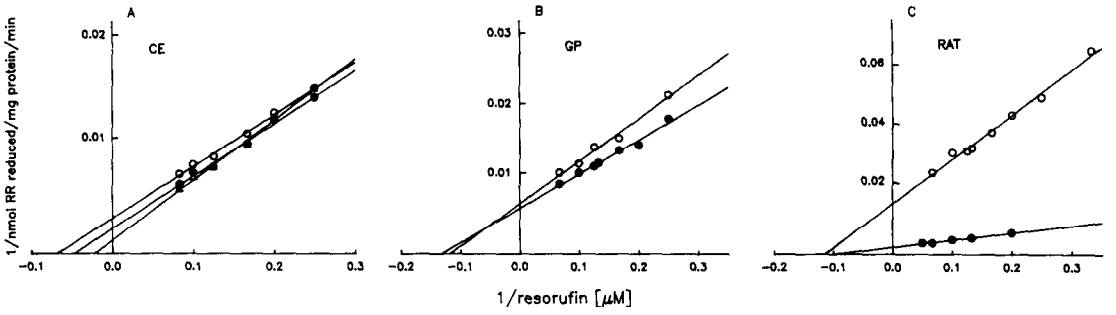

Fig. 4. Double-reciprocal plots for composite data for resorufin reduction by liver cytosols. (A) Chick embryo (CE); (B) guinea pig (GP); and (C) rat. Control (○), TCDD, 1 nmol/egg, for chick embryo or 10 μg/kg for guinea pig and rat (●); TCDD, 5 nmol/egg, for chick embryo (△). Treatments, cytosol preparation and reactions were as described in Materials and Methods.

Table 2. K_m values for resorufin and V_{max} values for resorufin reduction by liver cytosols of control and TCDD-treated chick embryo, guinea pig and rat

Species	Dose of TCDD (μg/kg)	K_m (μM)	% of Control	V_{max} (nmol/mg protein/min)	% of Control
Chick embryo	0.0	17 ± 2 (9)	100	355 ± 28 (9)	100
	6.4	22 ± 2 (6)	129	474 ± 22* (6)	134
	32.2	52 ± 9† (5)	306	976 ± 123* (5)	275
Guinea pig	0.0	9 ± 1 (3)	100	159 ± 10 (3)	100
	10.0	8 ± 1 (4)	89	175 ± 11 (4)	110
Rat	0.0	8 ± 1 (4)	100	68 ± 28 (4)	100
	10.0	11 ± 2 (3)	138	482 ± 71† (3)	709

Resorufin reduction was measured in liver cytosol from 19-day-old White Leghorn chick embryos, 300–350 g male Hartley guinea pigs, and 150–200 g male Sprague–Dawley rats, treated with solvent (0.01 mL dioxane for chick embryo, corn oil for guinea pig and rat) or TCDD in the same solvent, as described in Materials and Methods. The dose of TCDD is in μg/kg egg for chick embryo and μg/kg body weight for guinea pig and rat. Resorufin reduction was measured as described in Materials and Methods. Values for K_m and V_{max} (nmol resorufin reduced per mg cytosolic protein per min) are reported as means ± SEM. The numbers in parentheses represent the number of separate experiments (pools of 4–6 livers for chick embryo and individual livers for guinea pig and rat). For each species, the values for the TCDD-treated groups as a percent of the values for controls (treated with solvent alone) are also shown.

*, † P values compared to controls of the same species: * $P < 0.01$ and † $P < 0.05$.

isozyme plus an inhibitor.) In the guinea pig, TCDD at 10 μg/kg, ten times the LD_{50} [21], did not alter significantly the K_m for resorufin or the V_{max}. The same dose of TCDD in the rat (less than half the rat LD_{50}) did not affect the K_m for resorufin but increased the V_{max} 7-fold over control values ($P < 0.05$).

TCDD when present *in vitro* at 1.9 nM (ten times the concentration that would be expected in liver for a dose of 5 nmol/egg evenly distributed throughout the egg) together with cytosol, resorufin at 10 μM and NADPH at 1 mM had no effect on the resorufin reduction rate, indicating that TCDD did not affect resorufin reduction directly.

DT-Diaphorase measured by DCPIP reduction. DCPIP was reduced by chick embryo liver cytosol. The reduction was supported equally by NADPH and NADH and was inhibited by dicumarol. At 60 μM DCPIP, a non-limiting substrate concentration for rat cytosol [1, 10], the rates were the same or lower than in rat cytosol. Our values for DCPIP reduction by rat liver cytosol were within the range reported by others [10, 22, 23]. Also, as reported, the rate in rat cytosol was maximum at 60 μM DCPIP but in chick embryo cytosol (Fig. 5A) it was only beginning to reach a plateau at 180 μM, the highest concentration we could test because of the high absorbance of DCPIP. By double-reciprocal plots the K_m values (μM) for DCPIP were 32 and 67 and the V_{max} values (nmol DCPIP reduced per mg cytosolic protein per min) were 421 and 849 for the rat and chick embryo respectively (Fig. 5B). Thus, at non-limiting concentrations of DCPIP or resorufin, DT-diaphorase activity was greater in chick embryo than in rat liver cytosol. The degree of induction by TCDD was also similar with DCPIP or resorufin as substrates (in the rat, for TCDD at 10 μg/kg, 6-fold with DCPIP and 7-fold with resorufin; in chick embryo, at 6.4 μg TCDD/kg egg, 1.3-fold for both substrates, and at 32.2 μg, 1.9- and 2.75-fold with DCPIP and resorufin respectively.)

DT-diaphorase compared to 7-ethoxyresorufin deethylase in chick embryo, guinea pig and rat liver. Table 3 shows 7-EROD activity in microsomes from the livers for which cytosolic DT-diaphorase is shown in Table 2. The basal (control) level of 7-EROD (nmol resorufin formed per mg microsomal protein per min) was lowest in chick embryo, five times higher in rat and 4-fold higher in guinea pig than rat.

Table 3. Effect of TCDD on 7-ethoxyresorufin deethylase activity in liver microsomes of chick embryo, guinea pig and rat: comparison with resorufin reduction in cytosol

Species	Dose of TCDD (μg/kg)	7-EROD (nmol/mg protein/min)	Enzyme induction 7-EROD (treated/control)	Resorufin reduction
Chick embryo	0.0	0.05 ± 0.01 (9)		
	6.4	3.6 ± 0.9* (6)	72	1.3
	32.2	5.0 ± 1.8† (5)	100	2.8
Guinea pig	0.0	0.80 ± 0.08 (3)		
	10.0	6.0 ± 0.9† (4)	7.5	1.1
Rat	0.0	0.23 ± 0.06 (4)		
	10.0	21.6 ± 3.8† (3)	94	7.1

7-EROD was assayed in microsomes from the same livers in which resofurin reduction was assayed in cytosols (see Table 2). The reactions were performed as described in Materials and Methods. TCDD induction of 7-EROD and resorufin reduction (from Table 2) are compared in the last two columns. Values for 7-EROD (nmol resorufin formed per mg microsomal protein per min) are means ± SEM. The numbers in parentheses represent the number of separate experiments, as in Table 2.

*,† P values compared to controls of the same species: * $P < 0.001$ and † $P < 0.01$.

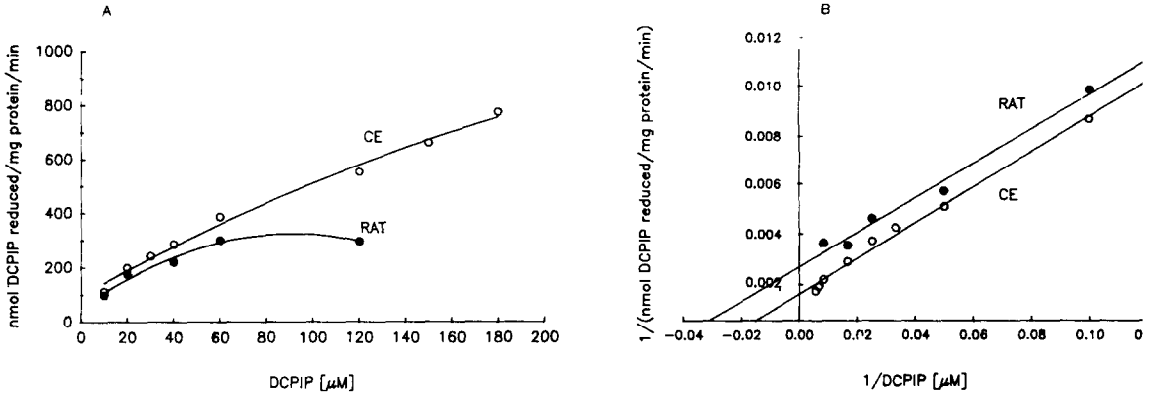

Fig. 5. Substrate–activity curve (A) and double-reciprocal plot (B) for DCPIP reduction by cytosols of control chick embryo (CE, ○) and rat (●). In addition to cytosol, reaction mixtures contained DCPIP, 10–180 μM; NADPH, 500 μM; and BSA, 0.07% (w/v). Reactions were carried out as described in Materials and Methods.

Our values for 7-EROD in the rat are essentially the same as those reported by Nims *et al.* [2]. TCDD at 10 μg/kg increased 7-EROD about 100-fold in the rat and only 7.5-fold in the guinea pig, about the same degree of hepatic induction as reported for guinea pig by 3-MC, another cytochrome P-448 type inducer [24]. In chick embryo the lower dose of TCDD increased 7-EROD about 72-fold and the higher dose about 100-fold (NS, $P = 0.5$). The induced levels of 7-EROD in guinea pig and chick embryo liver were similar (about 25% of the induced 7-EROD levels in rat).

The relationship of the degree of induction of DT-diaphorase to 7-EROD varied among the three species. Thus, doses of TCDD inducing more than three times as much DT-diaphorase in rat as in chick embryo liver induced 7-EROD comparably (about 100-fold) in both species. In the guinea pig a dose of TCDD without effect on DT-diaphorase significantly induced 7-EROD ($P < 0.01$).

Toxicity. TCDD at the doses used in these studes was toxic in the chick embryo and guinea pig but not in the rat. Chick embryo mortality was 12% (6 deaths out of 52 embryos) for controls; 29% (12 deaths out of 41 embryos, $P < 0.05$ compared to controls) for TCDD at 1 nmol/egg and 50% (25 deaths out of 50 embryos, $P < 0.001$) at 5 nmol, 7 days after exposure. All the TCDD-treated guinea pigs (10 μg/kg for 5 days) lost weight (mean decrease of 17%, $P < 0.02$), whereas the controls gained weight (mean increase of 10%). Thymus weights were decreased by 49% in the TCDD-treated guinea pigs (mean ± SEM, 0.55 ± 0.05 g for TCDD-treated vs 0.28 ± 0.04 g in controls, $P < 0.02$). In rats, in contrast, the same dose of TCDD (10 μg/kg), after 7 days, had no significant effect on body or thymus weights.

DISCUSSION

Chick embryo liver contains DT-diaphorase as measured by reduction of both resorufin and DCPIP. The reductions in chick embryo liver meet the criteria for a DT-diaphorase in that they are supported by

NADPH and NADH and inhibitable by dicumarol [1]. Chick embryo liver DT-diaphorase was entirely cytosolic in contrast to the rat, guinea pig and hamster liver enzymes which though primarily cytosolic are also present in microsomes and mitonchondria [1, 10,11]. Mouse liver [9] has also been reported to lack microsomal DT-diaphorase. The lack of activity in the mitochondrial fraction of chick embryo liver is consistent with the reported absence of DT-diaphorase in liver mitochondria of the pigeon (*Columba domestica*), another avian species [15].

Cytosolic resorufin reductase was similar in all three species in that the K_m values for resorufin varied less than 2-fold and there was substrate inhibition at resorufin concentrations over 20 μM. The chick embryo reductase, however, had a higher K_m for NADPH (10- and 3-fold higher than for rat and guinea pig respectively), a higher V_{max} (5- and 2-fold higher) and, as discussed below, different responsiveness to induction by TCDD. The greater reductase activity of chick embryo cytosol toward both DCPIP and resorufin demonstrates that DT-diaphorase is more active in chick embryo than in rat liver cytosol. The results also demonstrate the general utility of resorufin reduction as an assay for DT-diaphorase, extending its applicability to chick embryo and guinea pig liver.

For all three species, the catalytic efficiency of DT-diaphorase was greater toward DCPIP than toward resorufin as evidenced by ratios above unity for the rate of DCPIP to resorufin reduction (6.2, 2.4 and 1.5 for chick, rat and guinea pig*). This may reflect the higher redox potential of DCPIP (+0.217) than of resorufin (−0.051) [25] as a correlation between the redox potentials of DT-diaphorase substrates and their reduction rates has been observed [17]. The different ratios of DCPIP to resorufin reduction among species could reflect species differences in DT-diaphorase structure, as evidenced also by different purification characteristics of rat and mouse DT-diaphorase [17].

Another interesting aspect of the results is the relationship between DT-diaphorase and mixed-function oxidase induction. Since Lind and Ernster [26] first noted an association between DT-diaphorase and AHH induction by 3-MC in rat liver, DT-diaphorase induction has been widely viewed as part of the pleiotypic response to activation of the *Ah* receptor [12]. *Ah* receptor activation, however, can only partly account for DT-diaphorase induction as shown both by gene segregation studies [9] and the capacity of some chemicals that are not *Ah* receptor ligands to induce DT-diaphorase [27]. TCDD, the prototype *Ah* receptor ligand, would be expected to induce DT-diaphorase through the receptor. Also there is no evidence that TCDD is biotransformed into a metabolite characteristic of DT-diaphorase inducers acting independently of the receptor.

The ineffectiveness of TCDD in inducing DT-diaphorase in guinea pig liver [10] demonstrated, however, that *Ah* receptor agonists do not induce DT-diaphorase in all species. As *Ah* receptor binding leads to less mixed-function oxidase induction in liver of guinea pig than in other species, notwithstanding apparently similar *Ah* receptor number and binding affinity [24], the guinea pig is sometimes regarded as a special case. We confirmed using a different assay, that TCDD fails to induced DT-diaphorase in guinea pig liver. Our results show that this failure does not reflect the guinea pig's peculiar lack of expression of *Ah* receptor activation as a similar effect occurs in chick embryo liver which is highly responsive to mixed-function oxidase induction by *Ah* receptor activators [12–14, 28]. Thus, in chick embryo liver, a dose of TCDD that increased 7-EROD 72-fold barely increased DT-diaphorase (1.3 times control levels). The degree of association of DT-diaphorase and mixed-function oxidase induction by TCDD, therefore, appears to be species dependent.

The results could bear on the large and unexplained species differences in sensitivity to TCDD toxicity (oral LD_{50} values from 1 μg/kg in the guinea pig to 1000 μg/kg in hamster [21, 29]). Thus, we noted that TCDD at doses toxic to chick embryo and guinea pig caused little or no induction of DT-diaphorase in those species, whereas in rat a non-toxic dose induced DT-diaphorase much more. 7-EROD was also more induced in the rat than in the other species but high 7-EROD is not regularly associated with species resistance to TCDD toxicity. In hamster for example, which is even more resistant than rat to TCDD toxicity, TCDD induced 7-EROD less than in rat [24] but DT-diaphorase comparably [11]. The collective evidence thus suggests that there may be an inverse relationship among species for the degree of DT-diaphorase inducibility and sensitivity to TCDD toxicity and leads to the hypothesis that DT-diaphorase protects against TCDD toxicity. We suggest this with caution recognizing that the extent to which this association holds among other species, whether differences in liver induction of DT-diaphorase are reflected in other organs, or if liver DT-diaphorase can modulate toxicity in other organs are all unknown at this time.

The closeness of the constitutive (uninduced) levels of DT-diaphorase in chick embryo and TCDD-induced levels in rat liver cytosol could be seen to argue against a protective role of DT-diaphorase in TCDD toxicity. *In ovo*, however, DT-diaphorase activity toward some substrates may be lower than in cytosol *in vitro* because of competing oxidation of some substrates (as shown here for resorufin oxidation by chick embryo mitochondria and cytosol); or chick embryo may require higher NADPH levels to achieve maximal rates of reduction of some substrates as suggested by the 10-fold higher K_m for NADPH for resorufin reduction in chick embryo than in rat cytosol. Alternatively, the chick embryo could be adjusted to a high normal level of DT-diaphorase and after toxic stress may need even higher levels both to meet physiologic demands for DT-diaphorase (e.g. in Vitamin K-dependent carboxylation of coagulation factors and other proteins [30]) and to protect against quinone toxicity.

As TCDD is not metabolized to quinones, any protective effect of DT-diaphorase on TCDD toxicity would be unlikely to be exerted on TCDD *per se*. Quinones or quinone-like compounds, however, can come from normal dietary constituents (Vitamin

* The values for DCPIP reduction by guinea pig cytosol used in calculating this ratio were those reported by Beatty and Neal [10].

K and chloroplasts and mitochondria [31]) and from xenobiotics via peroxide [32] or NADPH-supported cytochrome P-450 oxidation [33]. From whatever source, quinones or quinone imines present in the body could be metabolized to potentially toxic semiquinones by NADPH cytochrome *c* reductase [3–7], or by cytochrome P-450 [34]. Thus TCDD, through induction of cytochrome P-450 and expression of its oxygenase and peroxidase activities might be expected to increase production by the body of semiquinone radicals. DT-diaphorase could potentially protect against toxicity from such products. Accordingly, the association of DT-diaphorase induction and species differences in resistance to TCDD toxicity and the possibility that DT-diaphorase protects against TCDD toxicity merit further investigation.

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