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Tryptophan 2,3-Dioxygenase in Chick Embryo Hepatocytes: Studies *in Ovo* and *in Culture* (42739)

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Abstract. Tryptophan dioxygenase is a hemoprotein in its active form, which has a relatively low affinity for heme. From previous studies in rats, the ratio of holoenzyme/total enzyme activity of tryptophan dioxygenase has been proposed to reflect the size of a "free" heme pool in hepatocytes. Chick embryo hepatocytes *in ovo* and in culture are other systems that have proven useful for study of hepatic heme metabolism and its control. Heretofore, there have been few studies of tryptophan dioxygenase activity in chick embryo hepatocytes. As part of studies on hepatic heme metabolism, using two different assays, we have measured tryptophan dioxygenase activity and percentage of heme saturation of the enzyme in chick embryo livers cells *in ovo* and in culture. One method of assay relies on endogenous formamidase to generate the final product, kynurenine, which is measured directly, whereas the other method uses a chemical hydrolysis step to form kynurenine which is further diazotized prior to measurement. The latter method is shown to be preferable for studies with chick embryo hepatocytes. In addition, we show that (i) tryptophan dioxygenase activity is present and can be increased by tryptophan and phenobarbital-like drugs in chick embryo hepatocytes *in ovo;* (ii) total enzyme activity falls markedly in cultured hepatocytes despite the presence of high concentrations of glucocorticoids in the culture medium; and (iii) under all conditions studied thus far in the cultures, the enzyme is nearly saturated with heme. Results are discussed in relation to regulation of heme metabolism in chick embryo hepatocytes.

L-Tryptophan 2,3-dioxygenase (EC 1.13.-11.11) catalyzes the breakdown of L-tryptophan to *N*-formylkynurenine. It is rate-controlling for tryptophan catabolism, found only in the soluble fraction of hepatocytes, and is subject to complex regulation, including induction by glucocorticoids, activation and stabilization by its substrate L-tryptophan, and activation by its co-factor heme (for review see (1)).

Assay of tryptophan dioxygenase activity typically involves quantifying kynurenine formed after incubation of an enzyme source with saturating concentrations of tryptophan and oxygen. Early measurements of activity were performed in the absence of heme (2). Later, total enzyme activity was assayed in the presence of heme (3–5). These methods rely on a presumed excess of endogenous formamidase activity in tissue extracts to metabolize *N*-formylkynurenine to kynurenine. Metzler *et al.* (6) have described an alternate and more sensitive assay in which the *N*-formylkynurenine, formed by the action of tryptophan dioxygenase, is nonenzymatically converted to kynurenine.

The enzyme requires heme for activity, but it does not bind heme as tightly as do some other cellular hemoproteins. Thus, under some conditions of assay, tryptophan dioxygenase in homogenates or extracts of liver has been found to exist in two forms: (i) as the active holoenzyme, containing heme, and (ii) as the inactive apo-protein, lacking heme (3, 4, 7). The ratio of holoenzyme/total enzyme activity (×100%) is also referred to as the percentage of heme saturation of trypotophan dioxygenase and, as first proposed by Badawy (8), has been suggested to reflect the size of a small but critical hepatocyte heme pool that regulates activity of 5-aminolevulinate synthase (and perhaps other heme-dependent cellular processes) (7, 9–13). Recently, however, the physiological relevance of low heme saturation of the enzyme has been questioned since studies with intact hepatocytes and homogenates of rat liver have indicated that tryptophan concen-

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trations in intact hepatocytes are sufficiently high to assure that the dioxygenase is always fully saturated with heme (14). With purified apo-tryptophan dioxygenase, the interdependence of tryptophan and heme binding has been demonstrated (2, 3); both heme binding and the activity of tryptophan dioxygenase increased with increasing tryptophan concentration.

Most prior studies of the dioxygenase and its relation to cellular heme status have been carried out in rats. However, cultures of rat hepatocytes undergo several changes (rapid loss of cytochrome *P*-450 (15), loss of normal inducibility of 5-aminolevulinate synthase (15), and decrease in 5-aminolevulinate dehydratase (16)) that limit their usefulness for studies of heme metabolism. In contrast, cultures of chick embryo hepatocytes retain these functions (17–20) and retain normal inducibility of at least two isoforms of cytochrome *P*-450 (21, 22). Accordingly, chick embryo hepatocytes *in ovo* and in culture are convenient and useful systems for studying hepatic heme metabolism and its regulation.

As a part of such studies, we have measured activities of tryptophan dioxygenase in chick embryo hepatocytes, first comparing the two methods of assay described above and then studying alterations in enzyme activity and in percentage of heme saturation produced by chemicals that influence cellular tryptophan dioxygenase or heme metabolism. Under all conditions studied thus far in chick embryo hepatocyte cultures, measurements of activity suggest that tryptophan dioxygenase is present predominantly as the active holoenzyme.

Methods and Materials. *Animals and injections.* Some 18-day-old chick embryos were treated with a suspension of L-tryptophan (50 mg/egg in 200 μl H_2O) or glutethimide (6 mg/egg in 200 μl dimethyl sulfoxide). Controls received the appropriate solvent. The livers were removed and thoroughly rinsed in 0.15 *M* NaCl.

Fed, male Sprague–Dawley rats (250 g) were killed by cervical dislocation and the livers were perfused thoroughly with 0.15 *M* NaCl.

Preparation of tissue fractions. Preparation of tissue fractions was performed on ice or at 4°C. Homogenates (10%, w/v) were prepared from livers in 0.25 *M* sucrose/0.02 *M* Tris–Cl, pH 7.4, or in 0.1 *M* potassium phosphate, pH 7.4. Homogenates were centrifuged 10 min × 10,000*g* to obtain the 10,000*g* supernatant fraction. In some cases, the 105,000*g* supernatant fractions and microsomes were prepared from the 10,000*g* supernatant fractions by centrifugation for 60 min at 105,000*g*. Microsomes were resuspended in sucrose–Tris buffer.

Hepatocyte cultures. Cultured hepatocytes were prepared from 16- to 18-day-old chick embryos as previously described (22) and maintained in Williams E medium (which contains 49 μM L-tryptophan) with added insulin (0.17 μM, first 24 hr only), T_3 (1.5 μM), and dexamethasone 0.76 μM). Approximately 2 hr was required to prepare the cell cultures.

After suitable intervals of culture and treatments, cells from two 10-cm plates (containing approximately 6 mg of total cell protein) were scraped into the medium, centrifuged for 2 min at 1000*g*, and resuspended in 1 ml of sucrose–Tris buffer. These cells were disrupted by sonication for 12 sec and centrifuged for 10 min at 10,000*g* to obtain the 10,000*g* supernatant fraction used for assays. Some of these samples were stored overnight at −80°C.

Tryptophan dioxygenase assays. Two methods were used for measuring tryptophan dioxygenase activity: Method 1 was a modification of the method of Metzler *et al.* (6) in which an azo-dye derivative of kynurenine (λ_{max} = 560 nm) was measured. In the original method, tryptophan and methemoglobin were included in the buffer used to homogenize the liver. However, in order to obtain the ratio of holoenzyme to total enzyme activity, we added tryptophan and methemoglobin at the start of the 37°C incubations. Final concentrations were L-tryptophan (3.9 m*M*), methemoglobin (2.1 μM), in 400 μl total volume, containing 0.2–0.5 mg of protein. The reaction was stopped by the addition of 600 μl of a 70% (w/v) perchloric acid:ethanol (1:1, v/v) mixture; further incubation at 37°C for 15 min completed the hydrolysis to kynurenine. Thereafter, the samples were rapidly cooled to −18°C in an ice–salt slurry, diazotized, and derivatized (6). Careful temperature control was essen-

tial to prevent high background absorbance from tryptophan destruction. The increase in absorbance between 30 and 90 min of incubation was used to calculate the activity. In all experiments, a zero time sample was processed to determine the blank value.

In some experiments, activity of tryptophan dioxygenase was determined by Method 2, namely that of Badawy and Evans (23). The 1.0-ml incubation mixture contained 3.3 m*M* L-tryptophan, 2.5 μM hemin (where indicated), and 2–3 mg protein of 10,000*g* supernatant fraction in 0.05 *M* sodium phosphate, pH 7.0. The formation of kynurenine at 37°C was followed with a thermostated dual wavelength spectrophotometer (SLM Aminco, DW2C, SLM Instruments, Champaign, IL).

Other assays. Concentrations of total cytochrome *P*-450 were measured on microsomes from the embryo liver by the method of Omura and Sato (24).

Heme oxygenase was measured by a modification of the method of Tenhunen *et al.* (25). The rate of bilirubin formation at 37°C was assessed by the increase in absorbance at 470 nm vs 540 nm. In addition to the sample (0.5 mg of microsomal protein), the final 1.0-ml reaction mixture contained 0.085 *M* potassium phosphate, pH 7.4, 3.8 m*M* deferoxamine, 25 μM hemin, 15 μM bovine serum albumin, biliverdin reductase (0.05 ml of a 105,000*g* supernatant fraction of 20% (w/v) homogenate of perfused rat liver), and 1 m*M* NADPH. The extinction coefficient (470–540 nm) for bilirubin obtained with a standard was 66 mM^{-1}.

Other analytical procedures. The concentrations of standard solutions of kynurenine were calculated from the absorbance at 365 nm using an extinction coefficient of 4.54 mM^{-1} (26). The extinction coefficient at 560 nm of the azo-kynurenine derivative (35.7 mM^{-1}) was determined with a kynurenine standard. Proteins were measured by the method of Lowry *et al.* (27), using bovine serum albumin as a standard.

Materials. Rats were from Charles River Laboratories (Raleigh, NC). Eggs were from Haley Farms (Canton, GA). Antibiotics, trypsin, glutamine, and Williams E medium were from GIBCO (Grand Island, NY). L-Tryptophan, hemin, NADPH, bovine serum albumin, and glutethimide were from Sigma Chemical Co. (St. Louis, MO). Deferoxamine was obtained from Ciba-Geigy through the Emory University Hospital Pharmacy. Other reagents were reagent grade from Sigma or Fisher Chemical (Phillipsburg, NJ).

Results and Discussion. *Activity of tryptophan dioxygenase in chick embryo liver.* In initial studies, activities of the dioxygenase, measured by Methods 1 and 2, were compared. The time course of the tryptophan dioxygenase assay in chick embryo liver for the two methods is shown in Fig. 1. For Method 1 the activity was nearly linear with time from 40 to 150 min. For Method 2 the

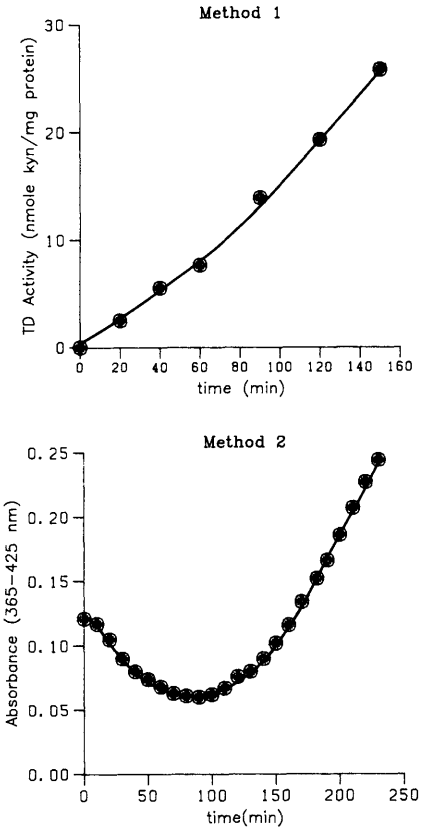

FIG. 1. Time course of tryptophan dioxygenase activity in two different assays. The reaction mixtures were as described under Methods and Materials. Method 1 with methemoglobin; the reactions were started at different times, and all were stopped at the same time. Method 2 with methemoglobin; continuous absorbance of the contents of a reaction cuvette. kyn, Kynurenine; TD, tryptophan dioxygenase.

TABLE I. EFFECT OF SAMPLE PREPARATION ON ACTIVITY OF TRYPTOPHAN DIOXYGENASE

Tissue	Pretreatment	Homogenizing buffer	Source of enzyme (supernatant)	Approximate preparation times[a]	Activity of tryptophan dioxygenase (pmole kynurenine/min × mg protein) Holoenzyme	Total enzyme	% Heme saturation	n
Chick	None	Sucrose–Tris	10,000*g*	30 min	272 ± 35	490 ± 9	55 ± 2	9
	None	Potassium phosphate	10,000*g*	30 min	103 ± 8	208 ± 16	50 ± 6	4
	None	Potassium phosphate	105,000*g*	2 hr	56 ± 17	99 ± 23	56 ± 8	3
	Tryptophan, 50 mg/egg, 16 hr	Sucrose–Tris	10,000*g*	30 min	1840 ± 127	2260 ± 150	69 ± 2	3
	Tryptophan, 50 mg/egg, 16 hr	Sucrose–Tris + 5 m*M* tryptophan	10,000*g*	30 min	2150 ± 29	2440 ± 32	88 ± 1	3
Rat	None	Potassium phosphate	10,000*g*	30 min	253 ± 18	464 ± 34	55 ± 4	3
	None	Potassium phosphate	105,000*g*	2 hr	44 ± 39	109 ± 21	40 ± 3	3

Note. The activity was measured by Method 1, as described under Methods and Materials. Values represent means ± SEM.

[a] Preparation times include the time from removal of the eggs from the incubator or from killing of rats to the beginning of the incubation.

apparent rate of product formation became linear after about 3.5 hr. The initial decrease in optical density by Method 2, observed with chick embryo liver preparations in our hands, did not occur with rat liver samples, although an initial decrease with rat liver samples had previously been reported by others (3, 28). Tryptophan dioxygenase activities in chick embryo livers, measured by Method 2, were consistently lower (59 ± 5%) than those measured by Method 1. The lower activity by Method 2 is probably due to (i) low formamidase activity in chick embryo liver (29), (ii) the large amount of time needed before the Method 2 assay became linear, and perhaps (iii) the presence of an unknown interfering substance or reaction. Thus, Method 1 is preferable for measuring activity of tryptophan dioxygenase in chick embryo hepatocytes and was used for all studies reported below.

As shown in Table I, values for tryptophan dioxygenase activity were sensitive to the sample preparation time and the homogenizing buffer. With chick embryo liver, higher values were obtained if the samples were prepared in sucrose–Tris, pH 7.4, than in 0.1 *M* potassium phosphate, pH 7.4. The lower values obtained for the 105,000*g* supernatant fraction, which were consistently observed, were probably due to longer preparation times.

The ratio of holoenzyme to total enzyme of control chick embryo liver homogenate was 0.50–0.55 (Table I), similar to values usually reported for rat liver (4, 8, 23). These studies were done with livers that had been rinsed free of blood prior to homogenization. In contrast, if livers were not thorougly rinsed, the ratio of holo- to total-enzyme activity was nearly unity, confirming an earlier report (29).

The apparent K_m for tryptophan in the presence of methemoglobin for the dioxygenase in chick embryo liver homogenate was 168 ± 9 μM (n = 3), similar to values of 150 μM previously reported for the enzyme from rat liver (2).

Effects of tryptophan. The effects of the administration of tryptophan on the activity of tryptophan dioxygenase of chick embryo livers are shown in Fig. 2. The activity increased for 16 hr, with a slight increase in percentage of heme saturation at 8 hr. The time response was slower than that in the rat, in which a maximal response was observed by 4 hr (4, 23). This may reflect a difference in the rate at which tryptophan enters hepatocytes following its injection into an egg, compared to that following intraperitoneal injection into a rat.

To investigate the effect of tryptophan during tissue preparation, tryptophan was added to the homogenizing buffer of livers

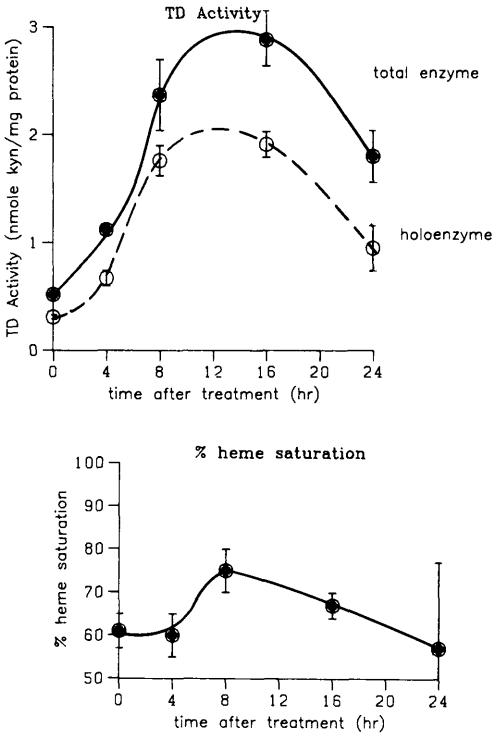

FIG. 2. Increase in activity of hepatic tryptophan dioxygenase produced by treatment of chick embryos with tryptophan. Embryos were injected with L-tryptophan (50 mg/egg). The assay was by Method 1, as described under Methods and Materials. Each point represents the mean ± SEM of three to seven measurements. The upper part of the figure shows activity of tryptophan dioxygenase, in the presence (● —— ●) and absence (O - - - O) of methemoglobin. The lower part of the figure shows the percentage of heme saturation of the enzyme. kyn, Kynurenine; TD, tryptophan dioxygenase.

from embryos pretreated with tryptophan. The heme saturation for samples prepared in homogenizing buffer containing 100 μM tryptophan was 92 ± 1%, whereas, without tryptophan in the buffer, the percentage of saturation was 69 ± 4% (Table I). In other experiments with control embryo livers, the effect of tryptophan in the homogenizing buffer was to increase heme saturation of the enzyme from about 50 to 90%. These results demonstrate a tryptophan-mediated increase in the degree of heme saturation of tryptophan dioxygenase activity in chick embryo liver consistent with the observation of the interdependence of heme binding and tryptophan concentration (2). Similarly, Salter and Pogson (14) noted that homogenization of rat liver in the presence of physiological concentrations of tryptophan resulted in 100% heme saturation of the enzyme. This and other evidence were interpreted to indicate that, in the hepatocytes of intact rats, the enzyme exists entirely as the holoenzyme. The physiological significance (or even occurrence) of heme unsaturation of dioxygenase remains unclear in chick or rat hepatocytes (14).

Activity of tryptophan dioxygenase in cultured chick hepatocytes. Activities of tryptophan dioxygenase in cultured chick embryo hepatocytes are shown in Fig. 3. The lower zero time activity in culture compared to activity of fresh homogenates of embryo liver is probably related to the 2-hr preparation time of cells for culture. After 24 hr of culture, little activity remained, indicating that the presence of dexamethasone (0.76 μM) was ineffective in maintaining activity. However, if 5 mM tryptophan was added at the time of plating, at 12 hr the activity was increased about 1.5-fold above that of the inoculum. Addition of tryptophan after 4 or 24 hr of culture increased activity of the dioxygenase above the corresponding control levels (Fig. 3). Thus, as in the intact embryo, tryptophan

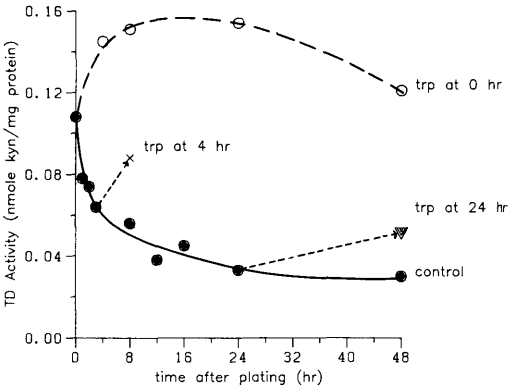

FIG. 3. Tryptophan dioxygenase activity in cultured chick embryo hepatocytes. Each point represents total enzyme activity for a separate plate. The Williams E medium, routinely used for maintaining the cultures, contains 49 μM tryptophan. L-Tryptophan (5 mM final concentration) was added to the culture medium of some plates at the times indicated. kyn, Kynurenine; TD, tryptophan dioxygenase; trp, L-tryptophan.

dioxygenase activity in cell cultures was increased in the presence of tryptophan.

Regardless of cell treatment, addition of methemoglobin to the assay had no effect on tryptophan dioxygenase activity in the cell culture system, suggesting that in the chick culture system all tryptophan dioxygenase exists as the holoenzyme. Thus, it is unlikely that heme saturation of tryptophan dioxygenase can be used as a measure of a regulatory heme pool in the culture system.

Effect of chemicals that increase cytochrome P-450 concentrations on heme metabolism and activity of tryptophan dioxygenase. Glutethimide increases cytochrome *P*-450, total cell heme, and activity of heme oxygenase in chick embryo hepatocytes *in ovo* and in culture (30). Effects of glutethimide on tryptophan dioxygenase activities of embryo livers *in ovo* are shown in Fig. 4. Within 2–4 hr of treatment, both holoenzyme and total enzyme activity increased; the heme saturation rapidly increased to almost 100% and remained elevated at 24 hr, even though the total enzyme activity fell to less than initial levels. Table II shows that similar results were obtained with two other chemicals that increase cytochrome *P*-450, namely mephenytoin and 20-methylcholanthrene. The percentage of heme saturation of the dioxygenase increased *before* either the concentration of cytochrome *P*-450 or heme oxygenase activity increased (Fig. 4 and Table II). Increased heme synthesis following drug treatment may increase the availability of heme for all hemoproteins and, in the case of tryptophan dioxygenase, thereby compensate for any lack of tryptophan. Alternatively, drug treatment may increase the intracellular free tryptophan concentration, thereby helping to increase the heme saturation of the dioxygenase. As already mentioned, there is an interdependence of heme and tryptophan binding to the dioxygenase during tissue preparation (4).

The rapid increase in apparent heme saturation following glutethimide administration (Fig. 4) further suggests that this chemical increases cellular heme synthesis before increased synthesis of the apo-protein moiety for cytochrome *P*-450 and/or assembly of new holo-cytochrome *P*-450 from heme and the apo-protein moiety occurs. The presence

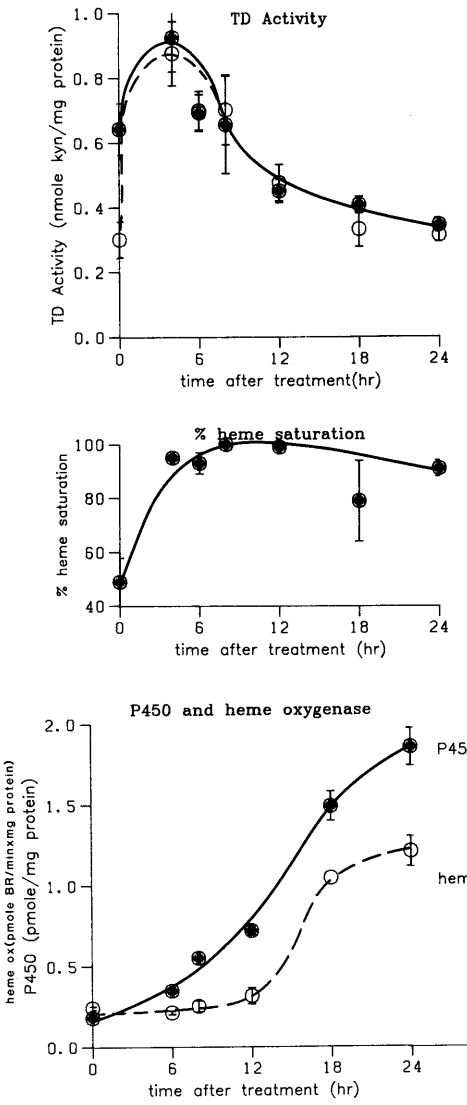

FIG. 4. Time course of the effects of glutethimide treatment of chick embryos on hepatic tryptophan dioxygenase, cytochrome *P*-450, and heme oxygenase. Embryos were injected with glutethimide, 6 mg/egg. At various times thereafter, embryos were killed, livers homogenized, and assays performed (Method 1), as described under Methods and Materials. Each point represents the mean ± SEM, $n = 3$–5. If not shown, error bars fell within the symbols. BR, Bilirubin; heme ox, heme oxygenase; kyn, kynurenine; P450, cytochrome *P*-450; TD, tryptophan dioxygenase.

of increased heme is known to be due to increased synthesis, rather than decreased degradation, since increased heme synthesis, in-

TABLE II. EFFECT OF CHEMICALS ON TRYPTOPHAN DIOXYGENASE, CYTOCHROME *P*-450, AND HEME OXYGENASE IN CHICK EMBRYO LIVER

Treatment	Time (hr)	Tryptophan dioxygenase (nmole kyn/min × mg protein) Holoenzyme	Total enzyme	Percentage of heme saturation	Cytochrome *P*-450 (nmole/mg protein)	Heme oxygenase (pmole BR/min × mg protein)
Control	—	0.19 ± 0.01	0.30 ± 0.03	63 ± 3	0.23 ± 0.03	0.26 ± 0.02
Mephenytoin	4	0.46 ± 0.04[a]	0.51 ± 0.05[a]	90 ± 4[a]	0.27 ± 0.04	0.23 ± 0.02
	16	0.18 ± 0.03	0.21 ± 0.03	86 ± 1[a]	1.44 ± 0.12[b]	0.38 ± 0.03[b]
20-Methyl-cholanthrene	4	0.40 ± 0.03[a]	0.40 ± 0.01[a]	100 ± 1[a]	0.24 ± 0.02	0.22 ± 0.02
	16	0.31 ± 0.05	0.37 ± 0.09	88 ± 8[a]	0.46 ± 0.03[b]	0.22 ± 0.03

Note. Chick embryos (18 days old) were injected with 0.2 ml dimethyl sulfoxide containing 6 mg mephenytoin or 1 mg 20-methylcholanthrene. Controls received solvent only. At the times indicated, embryos were killed, livers were removed, homogenized, and centrifuged and assays were performed as described under Methods and Materials. Results are expressed as means ± SEM, n = 3–4. BR, Bilirubin; kyn, kynurenine.

[a] Differs from control, $P < 0.05$.

[b] Differs from 4 hr and control, $P < 0.05$.

creased concentrations of cytochrome *P*-450 (30), and increased heme degradation occur in the presence of glutethimide and similar chemicals (manuscript submitted).

In conclusion, tryptophan dioxygenase in freshly prepared homogenates of chick embryo livers had an apparent activity and percentage of heme saturation similar to those of rat liver homogenates. In primary cultures of chick embryo hepatocytes, however, tryptophan dioxygenase was always completely saturated with heme. This behavior of the dioxygenase in cultured hepatocytes limits, and may even eliminate, its usefulness as a tool for studying regulatory heme pools in this experimental system. The regulation of tryptophan dioxygenase activity by tryptophan, heme, porphyrins (2), albumin (2), and other heme-binding proteins, indicates that caution should be exercised in interpreting the percentage of heme saturation of tryptophan diogenase as reflecting the size of a regulatory heme pool.

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