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Alterations in Hepatic Heme Biosynthetic Capability and Mixed Function Oxidase Activity During Ethionine Exposure in Rats

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SUMMARY

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These studies investigated the relationship between hepatic heme biosynthetic capability and mixed function oxidase activity in ethionine-treated rats. Animals fed a diet containing 0.25% D,L-ethionine for 2 to 3 months experienced up to a 65% decrease in hepatic aminopyrine demethylase activity and cytochrome P-450 and microsomal heme levels. These events were preceded by a loss of feedback repression of hepatic δ-aminolevulinic acid synthetase by heme and a 50% reduction in ferrochelatase activity as compared with that of untreated controls. A correlation between hepatic heme biosynthetic capability and mixed function oxidase activity was demonstrated in ethionine-fed animals using drugs and other agents which selectively alter heme biosynthetic pathway enzymes. These results indicate that specific changes in the regulation of hepatic heme biosynthesis occur during the early stages of ethionine exposure leading to decreased synthesis of microsomal heme and a relative deficiency of mixed function oxidase activities. It is suggested that heme may become rate-limiting with respect to microsomal mixed function oxidase activity under circumstances wherein heme biosynthesis has been chronically compromised.

INTRODUCTION

Recent studies from several laboratories have demonstrated the development of a deficiency in cytochrome P-450 and various other components of the microsomal mixed function oxidase system in livers of animals treated with ethionine (1), acetylaminofluorene (2), and other potential hepatocarcinogens (3-5) during prolonged exposure to these agents. Although the precise mechanism by which this phenomenon occurs has yet to be determined, these events are considered to be associated with the carcinogenic process and may represent one of the earliest recognizable biochemical lesions observed during the course of tumor development. However, the early appearance of this deficiency during exposure to a variety of agents of diverse chemical forms suggests also that this event might represent a nonspecific adaptive response of hepatocytes to continued injury by these substances. Further investigations are of interest to characterize more substantially this deficiency of hepatic mixed function oxidase activity by identifying the possible etiology of this phenomenon and by assessing the impact of these changes on drug metabolizing capability during the initial stages of chronic liver injury.

Studies of the possible mechanisms by which changes in the hepatic mono-oxygenase system are effected during exposure to

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agents such as ethionine have thus far failed to reveal the manner in which these alterations occur. Neither general inhibition of protein synthesis nor structural alterations of microsomal membranes has been associated with the decrease in mixed function oxidase activity induced by either ethionine or acetylaminofluorene (1, 2). In addition, changes in membranal lipid content are not considered to account for the changes observed (1). On the other hand, previous studies from this laboratory (6) have shown that the heme-mediated feedback regulation of ALA[1] synthetase, the rate-limiting enzyme in heme biosynthesis (7), is partially lost in livers of ethionine-fed rats; this observation suggests a possible association between impaired hepatic heme biosynthetic capability and the deficiency in mixed function oxidase levels which develops during exposure to this agent.

In the present studies, changes in the regulation of hepatic heme biosynthesis, both at the level of ALA synthetase and as affected by other enzymes of the heme biosynthetic pathway, were studied during the course of ethionine feeding in rats. A correlation between observed changes in heme biosynthetic capability and the development of mixed function oxidase deficiency was examined, and a functional association between these processes was demonstrated using drugs and other agents which specifically alter enzymes of the heme biosynthetic pathway and mixed function oxidase activity. In addition, the use of ethionine as a pharmacological tool for assessing drug metabolizing capability during chronic impairment of heme biosynthesis in mammalian liver is discussed in light of the current findings.

MATERIALS AND METHODS

Materials. D,L-Ethionine, cycloheximide, D,L-methionine and inosine were obtained from Sigma Chemical Co. Heme (Hemin chloride) was purchased from Calbiochem. Cobaltous chloride ($CoCl_2$) was obtained from K & K Laboratories. Mesoporphyrin-IX-dihydrochloride was purchased from Porphyrin Products. 3,5-Dicarbethoxy-1,4-dihydrocollidine was obtained from Eastman Chemical Co. Lubrol-WX was purchased from General Biochemicals. Allylisopropylacetamide was a gift of Hoffman La Roche, Inc. 4-Dimethylaminoantipyrine (aminopyrine) was purchased from Aldrich Chemical Co. Other chemicals were of reagent grade and were obtained from standard commercial sources.

Treatment of animals. Male Sprague-Dawley rats (CD strain) weighing 100–150 g at the beginning of the study were obtained from Charles River Laboratories and were individually housed in wire bottom cages. All animals were allowed food and water ad libitum throughout the investigation but were fasted for 24 hours immediately preceding each experiment. When drugs were given, animals were fasted for 24 hours prior to drug administration. Controls were maintained on a standard Wayne Lab-Blox diet (Allied Mills, Inc.) prepared in powdered form, whereas experimental animals received the same diet supplemented with 0.25% D,L-ethionine. Experimental rats were retained on the ethionine-supplemented diet for a maximum of eight months after which they were placed on the same ethionine-free diet as was fed to paired controls. Although body weights of experimental rats did not increase as rapidly as those of controls during the first eight months, liver to body weight ratios remained approximately equivalent in all animals throughout the investigation.

Drugs were administered to animals by intraperitoneal or subcutaneous injection at various times prior to sacrifice. Crystalline hemin was dissolved in a small volume of 0.01 M NaOH and adjusted to pH 7.5 with 0.01 M HCl in 0.9% NaCl to a final concentration of 10 mg/ml. Hemin was then administered by intraperitoneal injection at a dose of 20 mg/kg. $CoCl_2$ was dissolved in sterile isotonic saline to a concentration of 50 mg/ml and was administered at a dose of 50 mg/kg by subcutaneous injection. AIA[1] and DDC[1] were dissolved in a small quantity of 50% ethanol and also given by subcutaneous injection at doses of 300 or 500 mg/kg, respectively.

[1] The abbreviations used are ALA, δ-aminolevulinic acid: AIA, allylisopropylacetamide; DDC, 3,5-dicarbethoxy-1,4-dihydrocollidine; DTE, dithioerythritol.

Phenobarbital was administered intraperitoneally at a dose of 60 mg/kg.

Preparation of tissues. Animals were killed by decapitation. Livers were perfused *in situ* with cold 1.15% KCl and were then rapidly excised, minced and homogenized at 4° in 9 volumes of 0.25 M sucrose containing 0.05 M Tris-HCl buffer, pH 7.5, using a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Mitochondria were prepared from the homogenate as previously described (8).

The microsomal fraction was prepared from the 9,000 × *g* supernatant solution derived during preparation of mitochondria by centrifugation at 105,000 × *g* for 60 min. The final pellet was gently resuspended in 0.05 M Tris buffer so that the final suspension contained approximately 15 mg of microsomal protein per milliliter.

Histological evaluations. Histological examinations of liver sections taken from ethionine-treated rats were performed at bimonthly intervals during the course of exposure as well as at 12 and 18 months after treatment was begun. Following sacrifice, liver slices were excised and immediately fixed in a solution of 10% formalin containing 0.1 M potassium phosphate buffer, pH 7.0. Preparation and examination of all tissues including confirmation of the stages of tissue transformation during the course of neoplastic development were made according to the system of Farber (9) by the Laboratory of Environmental Biology and Chemistry, NIEHS. Hyperplastic nodules found in livers of rats fed ethionine-containing diets for 6 to 8 months were carefully dissected from surrounding liver and were prepared for tissue analysis as described above.

Assays of enzyme activities and other parameters. Mitochondrial ALA synthetase activity was measured as previously described (8) with the exception that beta-mercaptoethanol and EDTA were omitted from the incubation media. Reaction mixtures contained approximately 4 mg of mitochondrial protein per milliliter.

ALA dehydratase activity was measured in the 9,000 × *g* supernatant solution fraction of liver homogenates by a modification of the method of Gibson *et al.* (10) as described by Baron and Tephly (11).

Uroporphyrinogen I synthetase was assayed by a modification of the method of Levin and Coleman (12) wherein the rate of disappearance of porphobilinogen was measured. Details of this modification have been described previously (13).

Mitochondrial ferrochelatase activity was measured by a previously published modification (14) of the method of Porra (15) using mesoporphyrin-IX-dihydrochloride as substrate. Reaction mixtures contained approximately 4 mg of mitochondrial protein per milliliter.

Aminopyrine N-demethylase activity in microsomal preparations was measured by a modification of the procedure of Orrenius (16) as described by Lucier *et al.* (17). Reaction mixtures contained approximately 3 mg of microsomal protein per milliliter.

Cytochrome P-450 levels were determined on a Beckman Acta III recording spectrophotometer by measurements of carbon monoxide difference spectra following reduction with dithionite (18). An extinction coefficient of 91 $mM^{-1}cm^{-1}$ was assumed for the difference in absorption between 450 and 490 nm.

Microsomal hemes were quantitated by the pyridine hemochromogen method as described by Falk (19). Heme content was calculated from the difference in absorption between 541 nm and 557 nm using a value of 20.7 $mM^{-1}cm^{-1}$ as the extinction coefficient.

Protein determinations were made by the method of Lowry *et al.* (20) using bovine serum albumin (fraction V) as standard.

Partial purification of hepatic ALA synthetase from control and ethionine-fed rats. ALA synthetase from control and ethionine-fed rat liver was partially purified by a modification of previously described procedures (21). Five control and five ethionine-fed rats (8 weeks) were treated 24 hours prior to sacrifice with AIA (300 mg/kg). Livers from each group were removed, weighed, pooled and homogenized in a Waring blender in 9 volumes of 0.25 M sucrose containing 0.05 M Tris-HCl buffer, pH 7.5, and 0.1 mM pyridoxal phosphate. Mitochondrial preparations were washed and suspended in 1.5 volumes of Tris buffer

containing 0.1 mM pyridoxal phosphate, 1 mM DTE[1] and 0.9% NaCl. An aqueous solution of Lubrol-WX was then added to a final concentration of 0.6%, and suspensions were stirred at 4° for 1 hour. Following centrifugation for one hour at 105,000 × *g*, supernatant solutions were brought to 55% with respect to ammonium sulfate and stirred at 4° for 15 min. Mixtures were then centrifuged for 15 minutes at 10,000 × *g*, and pellets were suspended in a small volume of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 mM pyridoxal phosphate, 1 mM DTE and 0.9% NaCl. Solutions were then placed on columns of Sephadex G-200 equilibrated with the same buffer, and 5 ml fractions were collected. This procedure typically resulted in a 3 to 5 fold increase in specific activity of the enzymes as compared with those of original mitochondrial preparations.

RESULTS

Preliminary studies from this laboratory have demonstrated a partial loss of mixed function oxidase activity in livers of rats fed a diet containing 0.25% ethionine for extended periods (22). In the present investigation studies were performed to ascertain the time course of the development of this deficiency with respect to cytochrome P-450 levels and aminopyrine N-demethylase activity during chronic ethionine feeding. These results are shown in Figs. 1 and 2. During the early stages of treatment, a deficiency in mixed function oxidase capability becomes a characteristic property of the entire liver, with decreases in both cytochrome P-450 (Fig. 1) and aminopyrine demethylase (Fig. 2) levels to 35 to 60% of control values being observed as early as 1 to 2 months following initial exposure to the agent. Subsequently, the decreases in cytochrome P-450 content and aminopyrine demethylase activity become primarily associated with the hyperplastic nodules, and finally with the tumors which eventually develop in the livers of these animals. The deficiency in cytochrome P-450 and N-demethylase activity is accompanied by a progressive decrease in microsomal heme levels during the first three months following initial exposure to ethionine (Fig. 3).

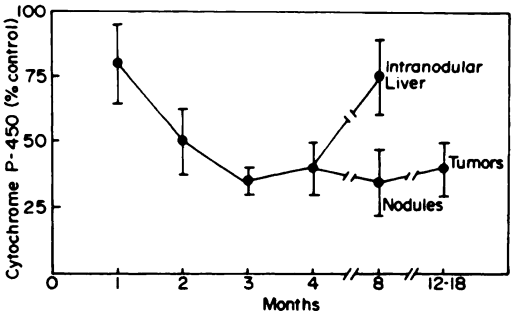

FIG. 1. *Time course of changes in hepatic microsomal cytochrome P-450 levels during ethionine feeding in rats*

Animals were killed at the times indicated after the beginning of ethionine exposure. In this and subsequent figures values represent the mean ± SEM of at least five experiments. Livers of four rats were pooled for each experimental value. Cytochrome P-450 levels at the beginning of the experiment were 1.25 ± 0.35 nmoles/mg protein.

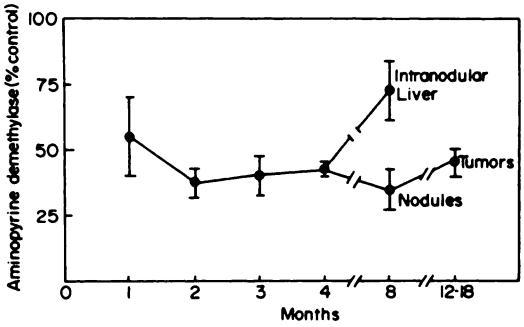

FIG. 2. *Time course of changes in hepatic microsomal aminopyrine demethylase activity during ethionine feeding in rats*

Enzyme activity at the beginning of the experiment was 4.65 ± 0.38 nmoles H_2CO/mg protein/min.

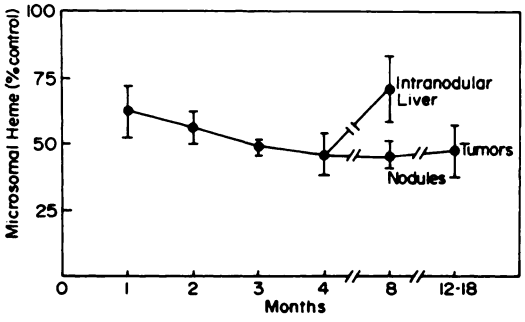

FIG. 3. *Time course of changes in hepatic microsomal heme levels during ethionine feeding*

Heme levels at the beginning of the experiment were 1.34 ± 0.08 nmoles/mg protein.

In order to examine the relationship between the observed deficiency of microsomal heme and mixed function oxidase levels and heme biosynthetic capability during the early stages of ethionine feeding, studies were next performed to investigate possible alterations in the regulation of heme biosynthesis in livers of rats fed ethionine for up to four weeks. Initial studies were directed toward examination of the regulation of ALA synthetase, which, in normal adult liver, is the rate-limiting enzyme in this process (7). At least three mechanisms of regulation of ALA synthetase, mediated by the end-product, heme, have been reported. These are schematically illustrated in Fig. 4. The principal mode of regulation is considered to involve feedback repression of *de novo* synthesis of ALA synthetase by heme (23). In addition, heme is thought to influence ALA synthetase activity in the cell through a process involving direct feedback inhibition of the enzyme (24, 25). Finally, Hayashi *et al.* (26) have shown that heme prevents the physiological activation of ALA synthetase *in vivo* by inhibiting its incorporation into the mitochondria, subsequent to its synthesis in the endoplasmic reticulum. All three modes of regulation were investigated in these studies.

Results of experiments designed to examine the role of heme in the feedback repression of the *de novo* synthesis of ALA synthetase are given in Fig. 5. In these studies the effects of ethionine exposure on both the induction and repression of ALA synthetase were investigated, as these processes are considered to proceed through a common mechanism. Administration of either AIA or DDC produced a 3.5 to 5-fold induction of ALA synthetase in both control and ethionine-fed animals. In contrast, ethionine-fed rats were refractory to the effects of heme, given either alone or in combination with one of the inducing agents. Whereas heme depressed ALA synthetase levels by over 50% of control values in untreated rats and substantially prevented enzyme induction by either AIA or DDC, heme produced neither effect in livers of ethionine-fed animals.

Refractoriness of ALA synthetase to supression by heme did not appear to result from changes in the physical properties of ALA synthetase in livers of ethionine-fed rats or to alterations in the responsiveness of ALA synthetase to heme *in vitro*. As indicated in Table 1, both the specific activity and the half-life of ALA synthetase, determined after injection of animals with cycloheximide as previously described (8), were similar in livers of control and ethionine-fed rats. In addition, enzymes which were partially purified from livers of control and ethionine-fed animals behaved similarly with respect to substrate affinities for glycine and succinyl coenzyme A, and were inhibited to an equal extent by heme.

The effects of heme in the mitochondrial incorporation of ALA synthetase in livers of control and ethionine-fed rats were also

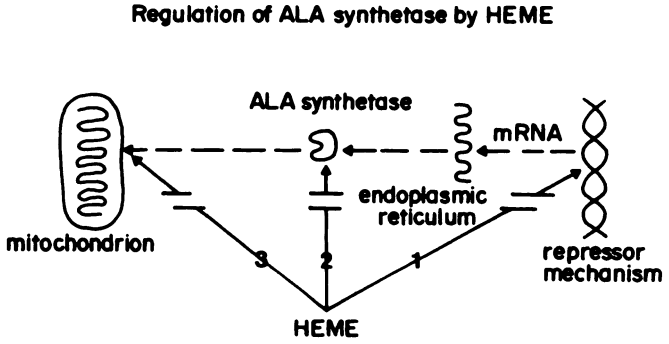

FIG. 4. *Schematic representation of three proposed mechanisms of the regulation of ALA synthetase activity by heme*

1. Repression of *de novo* enzyme synthesis. 2. Direct enzyme inhibition. 3. Suppression of incorporation of newly formed enzyme into mitochondria.

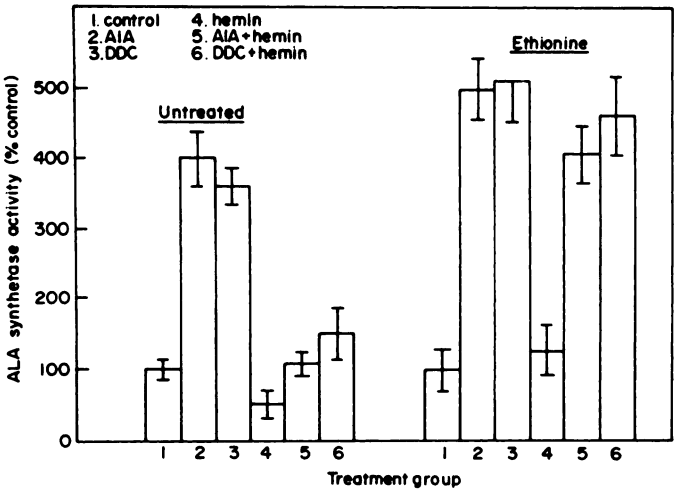

FIG. 5. *Effects of heme and AIA or DDC on hepatic ALA synthetase activity in untreated and ethionine-fed (4 weeks) rats*

TABLE 1

Properties of hepatic ALA synthetase from untreated and ethionine-fed rats

Property	Untreated	Ethionine-fed
In Vivo		
Specific activity (nmoles/mg/hr)	0.62 ± 0.02	0.64 ± 0.03
Half-life (min)	72	64
In Vitro		
Km glycine (M)	5.9×10^{-3}	6.0×10^{-3}
Km succinyl CoA (M)	1.6×10^{-5}	1.8×10^{-5}
Hemin inhibition (%)		
1 μM	15	13
10 μM	50	58
100 μM	92	89

similar (Fig. 6). When hemin was given to rats by i.v. injection two hours following induction of ALA synthetase by AIA, mitochondrial ALA synthetase activity declined by an equal amount in both groups and accumulated to an equivalent extent in the postmitochondrial supernatant fractions when measured over a time course of four hours.

These results suggest that a primary deficiency in the regulation of ALA synthetase develops at the level of feedback repression of *de novo* enzyme synthesis by heme during ethionine-feeding. This suggestion is substantiated by the observed lack of change in the specific activity of ALA synthetase both after depletion of microsomal heme levels and following injection of hemin at doses which produce a 50% decrease in ALA synthetase activity in control livers. In order to document the time course of this phenomenon studies were next performed to ascertain the duration of exposure required to produce refractoriness of ALA synthetase to regulation by heme during the course of ethionine feeding. These studies indicated that refractoriness equivalent to that seen in 4 week-exposed animals quickly became evident as early as 10–12 days following initial exposure to ethionine in the diet. Heme neither repressed nor prevented induction of ALA synthetase by AIA at this stage of exposure. In contrast, in animals given a single intraperitoneal injection of ethionine (250 mg/kg) 24 hours before treatment, or fed ethionine in the diet for one week, the responsiveness of ALA synthetase to hemin injection was similar to that observed in untreated controls.

The effects of ethionine feeding on ALA synthetase regulation by heme were completely reversible during the first 12 weeks of treatment if animals were removed from exposure to ethionine for at least one week after treatment. However, reversibility of these effects could not be attained subsequent to the appearance of hyperplastic nodules which developed during a prolonged course (4 to 5 months) of exposure.

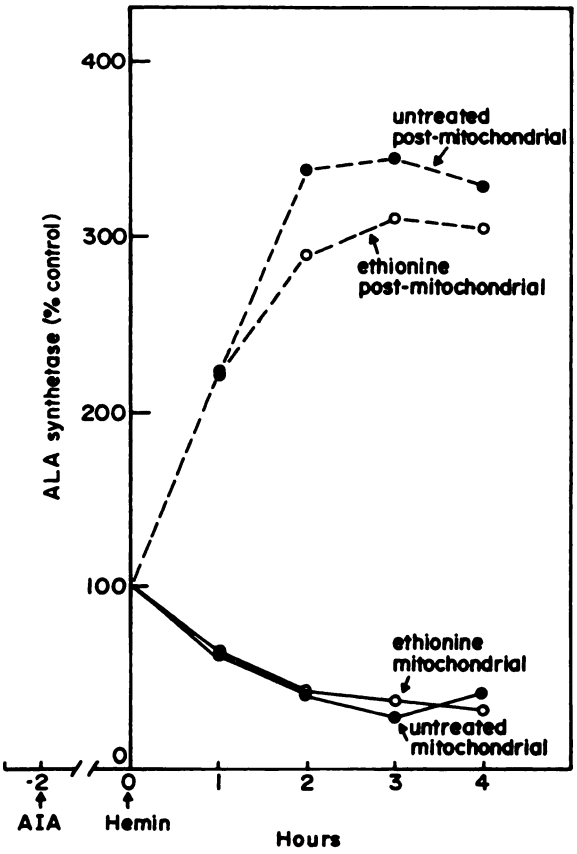

FIG. 6. *Effects of heme on the mitochondrial incorporation of ALA synthetase in untreated and ethionine-treated rats*

Hemin (20 mg/kg) was administered two hours following treatment of animals with AIA (300 mg/kg). Animals were killed at the times indicated.

In addition, a partial protection against the effects of ethionine on the feedback regulation of ALA synthetase by heme was observed when rats were fed ethionine-containing diets fortified with either 0.8% methionine or 0.5% inosine for four weeks. In these cases, heme was 50 to 75% as effective in either repressing ALA synthetase or in preventing its induction by AIA as was seen in untreated controls.

The observed refractoriness of ALA synthetase to regulation by heme in ethionine-fed rats suggests the possibility that some enzyme other than ALA synthetase may become rate-limiting in heme biosynthesis in livers of these animals. In order to investigate this possibility, other enzymes of the heme biosynthetic pathway, some of which are known to limit heme biosynthetic capability under various pharmacologic or pathologic circumstances (11, 27–29), were examined in rats treated with ethionine for four weeks. Results of these experiments, given in Table 2, indicated that no significant ($p < 0.05$) changes occurred in the activities of either ALA dehydratase or uroporphyrinogen I synthetase in livers of ethionine-exposed animals. In contrast, the activity of ferrochelatase, the mitochondrial enzyme which catalyzes the final step in the formation of heme, was depressed by as much as 50% in comparison with that of controls. As in the case of the refractoriness of ALA synthetase to heme, this deficiency also developed rather quickly over a time course of 10–12 days following initial exposure to ethionine. On the other hand, 3 to 4 weeks was required prior to a decrease in

mixed function oxidase activity or microsomal heme levels in ethionine-fed rat liver.

These results suggested that a primary deficiency in ferrochelatase activity which might substantially limit heme production evolves during the early stages of ethionine exposure. This event, coupled with the observed lack of responsiveness of ALA synthetase to feedback regulation by heme, suggests a situation wherein the biosynthesis of heme becomes rate-limiting in the synthesis of cytochrome P-450 and thus of mixed function oxidase activity during ethionine exposure. In order to assess further this possibility experiments were performed to examine the correlation between heme biosynthesis and mixed function oxidase activity in ethionine-fed rats using drugs which selectively alter various components of the heme biosynthetic pathway. Results of these experiments are given in Table 3. Untreated values represent the actual activities of ALA synthetase, ferrochelatase and aminopyrine demethylase, and cytochrome P-450 levels, measured in livers of rats fed ethionine for four weeks. Treatment of such animals with AIA, a drug which induces ALA synthetase but not ferrochelatase, increased ALA synthetase activity by over 3-fold but had no effect on cytochrome P-450 levels or aminopyrine demethylase activity. On the other hand, DDC, an agent which inhibits ferrochelatase (29, 30) but induces ALA synthetase, caused a decrease in cytochrome P-450 levels and aminopyrine demethylase activity, despite a 5-fold induction of ALA synthetase. Cobalt chloride, which both inhibits ferrochelatase (14, 31) and promotes the intracellular catabolism of heme (32, 33), also caused a decrease in mixed function oxidase activity. Phenobarbital, which in-

TABLE 2

Activities of hepatic heme biosynthetic pathway enzymes in control and ethionine-fed rats

Values represent the mean ± SEM of at least five experiments. Livers of four rats from each treatment group were pooled for each experimental value. Enzyme assays were performed as described in MATERIALS AND METHODS.

	ALA synthetase	ALA dehydratase	Uroporphyrinogen I synthetase	Ferrochelatase
	(nmoles ALA/mg prot/hr)	*(nmoles PBG/mg prot/hr)*	*(nmoles PBG/mg prot/hr)*	*(nmoles mesoheme/mg prot/hr)*
Control	0.529 ± 0.09	8.41 ± 0.64	8.13 ± 0.59	2.92 ± 0.25
Ethionine-fed	0.626 ± 0.11	7.99 ± 0.53	9.35 ± 0.72	1.74 ± 0.21

TABLE 3

Effects of agents which alter heme biosynthetic capability on mixed function oxidase activity in livers of ethionine-fed rats

Animals were fed an ethionine-fortified diet for four weeks. Drugs were administered 16 hours prior to sacrifice. Subcellular fractions were prepared for assays as described in MATERIALS AND METHODS. Values represent mean ± SEM of at least four experiments. Livers of four animals were pooled for each experimental value. Phb = phenobarbital.

Treatment	ALA synthetase	Ferrochelatase	Cytochrome P-450	Aminopyrine demethylase
	(nmoles ALA/mg prot/hr)	*(nmoles mesoheme/mg prot/hr)*	*(nmoles/mg protein)*	*(nmoles H_2CO)/mg prot/min)*
1. Untreated	0.64 ± 0.09	1.85 ± 0.11	0.52 ± 0.02	4.65 ± 0.38
2. AIA (300 mg/kg)	2.04 ± 0.15[a]	1.67 ± 0.23	0.48 ± 0.05	4.36 ± 0.54
3. DDC (500 mg/kg)	3.07 ± 0.19[a]	1.11 ± 0.09[a]	0.29 ± 0.03[a]	2.21 ± 0.42[a]
4. $CoCl_2$ (50 mg/kg)	0.62 ± 0.03	1.47 ± 0.17[a]	0.38 ± 0.02[a]	2.65 ± 0.23[a]
5. Phb (60 mg/kg)	0.90 ± 0.21	2.63 ± 0.28[a]	0.73 ± 0.11[a]	6.51 ± 0.43[a]
6. Phb + $CoCl_2$	1.01 ± 0.25[a]	1.98 ± 0.16	0.46 ± 0.08	3.72 ± 0.45

[a] $p < 0.05$ vs. control.

duces both ferrochelatase as well as ALA synthetase in normal liver (34), caused an increase in cytochrome P-450 content and N-demethylase activity. However, phenobarbital given in combination with cobalt chloride was ineffective with respect to altering monooxygenase levels. Phenobarbital is also known to induce *de novo* cytochrome P-450 apoprotein synthesis (35).

Experiments were also performed to ascertain the effects of ethionine on ferrochelatase activity and cytochrome P-450 levels *in vitro*. In these experiments ethionine was dissolved in 0.2 N NaOH and was added directly to either ferrochelatase assay reaction mixtures or to microsomal suspensions utilized in cytochrome P-450 determinations. Final concentrations were 0, 0.1, 1.0 or 10 mg ethionine/ml. Assays were carried out as described in MATERIALS AND METHODS. Results of these experiments showed that ethionine did not directly inhibit ferrochelatase or reduce cytochrome p-450 levels under the experimental assay conditions.

DISCUSSION

Numerous studies in recent years have sought to elucidate the nature of the association between the biosynthesis of heme and the synthesis and functional activation of various hemoproteins and cytochrome-dependent processes in mammalian tissues. Evidence has been presented to suggest that synthesis of protein, rather than heme, is the rate-limiting event in the biosynthesis of microsomal cytochrome P-450 in normal adult liver (36). In contrast, the availability of heme may limit the synthesis and functional activation of cytochrome oxidase or other hemoproteins in both adult (37, 38) and fetal (39) mitochondria. The results of the present study suggest that, while protein synthesis may restrict mixed function oxidase levels in adults under normal circumstances, heme synthesis becomes the rate-limiting event in this association under conditions wherein heme biosynthesis has been chronically compromised. This hypothesis is supported both by time course studies, which demonstrated a temporal relationship between alteration in heme synthesis and the decline in functional mixed function oxidase levels, and by the observation that specific drug-induced fluctuations in heme synthesis produce concomitant changes in monooxygenase activity in livers of ethionine-fed rats.

The observed deficiency in heme biosynthetic capability which develops during ethionine feeding in rats is characterized by alterations in at least two components of the heme biosynthetic pathway in the liver. Foremost is the impairment of regulation of ALA synthetase by the end product, heme. Although the underlying mechanism of this phenomenon has not been precisely defined in these studies, it seems reasonable to conclude that it is not associated with changes in the physical properties of ALA synthetase nor with alterations in the capability of heme to modulate the mitochondrial incorporation and, hence, the physiological activation of the enzyme *in vivo*. On the other hand, an ethionine-induced change in the DNA-mediated process through which heme represses *de novo* synthesis of ALA synthetase could account for the observed changes. Since ethionine does not seem to affect the mechanism through which ALA synthetase is induced, it is possible that the observed refractoriness to heme is mediated via inhibition by ethionine of a specific aporepressor protein to which heme must bind in order to exert regulatory control of ALA synthetase synthesis. This hypothesis is consistent with the known property of ethionine as a specific protein synthesis inhibitor in mammalian cells (5, 40, 41). Alternatively, ethionine may act to inhibit the process of entry of heme into hepatocellular nuclei, thereby reducing its efficacy as a physiological repressor of ALA synthetase synthesis.

The present studies have also indicated that ethionine exerts a selective inhibitory effect with respect to ferrochelatase in comparison with other enzymes of the heme biosynthetic pathway which were investigated. Whether this effect is exerted at the level of synthesis of ferrochelatase or via direct inhibition of the enzyme is not clear. Since ethionine did not alter ferrochelatase activity *in vitro*, it is possible that inhibition of this enzyme is effected by metabolites of the parent compound produced by meta-

bolic activation of ethionine *in vivo*. The time course of inhibition of ferrochelatase during ethionine exposure suggests an effect at the level of enzyme synthesis which requires a specific time course of exposure to develop. In addition, this effect appears to be incomplete or partially reversible, since the enzyme remains susceptible both to induction by phenobarbital and to further inhibition by $CoCl_2$ and DDC. It is plausible, therefore, that this decrease is mediated via a mechanism similar to that postulated to be responsible for the altered regulation of ALA synthetase by heme.

The question as to whether ethionine or some metabolite thereof mediates the destruction of heme *in vivo* cannot be ascertained from the results of the present investigations. This point is of interest, however, in light of recent studies (42) reporting the destruction of the heme of cytochrome P-450 *in vitro* by metabolites of vinyl chloride as well as other agents. In light of the observed time course required for the loss of cytochrome P-450 levels during ethionine feeding in the present studies, as well as the lack of effect of ethionine on P-450 levels *in vitro*, it is doubtful that such a mechanism is operating in this case. However, a further investigation of this possibility using a system designed to assess the effects of ethionine and its possible metabolites on purified cytochrome P-450 and other components of the mixed function oxidase system *in vitro* would be useful in providing further insight into the mechanisms of the ethionine-mediated decrease in hepatic monooxygenase activity during prolonged exposure to this agent.

The relationship of the observed changes in the regulation of hepatic heme biosynthesis and the concomitant deficiency of mixed function oxidase activity to the induction of hepatocellular carcinoma or other forms of chronic liver disease is not yet known; however, the present findings may be of importance with regard to understanding the role and functioning of the hepatic monooxygenase system in the early stages of these processes. Since the alterations in both heme biosynthesis and mixed function oxidase activity become characteristic properties of the hyperplastic nodules and of the tumors which eventually develop in the livers of ethionine-fed rats, these changes may represent one of the initial biochemical events which occur during the course of neoplastic transformation in the liver. In this regard the development of deficiencies in heme biosynthetic capability and, consequently, mixed function oxidase activity may represent an early adaptive response of the liver cell which serves to protect the organ from cytotoxicity during prolonged exposure to agents which are normally activated via the mixed function oxidase system.

In conclusion, the present studies demonstrate a deficiency in heme biosynthetic capability which develops concomitantly with a decrease in microsomal monooxygenase activity during the early stages of ethionine exposure in rats. This deficiency is characterized by both a partial loss of feedback regulatory control of ALA synthetase by heme and a decreased activity of ferrochelatase in livers of ethionine-fed rats. Both temporal and drug-specific correlations between changes in heme biosynthesis and mixed function oxidase activity were demonstrated, suggesting that heme may become rate-limiting in the synthesis and functional activation of various components of the monooxygenase system under circumstances wherein heme biosynthetic capability is chronically compromised. In this regard, liver of ethionine-fed rats may provide an interesting model system in which to study the fundamental role of heme in the development and maintenance of mixed function oxidase capability and in which to assess the biochemical consequences of chronic impairment of heme biosynthesis on the disposition of drugs and other agents which are normally metabolized via the hepatic monooxygenase system.

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