Effect of 3-Amino-1,2,4-triazole on the Stimulation of Hepatic Microsomal Heme Synthesis and Induction of Hepatic Microsomal Oxidases Produced by Phenobarbital

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SUMMARY

3-Amino-1,2,4-triazole inhibits rat hepatic δ-aminolevulinic acid dehydratase (EC 4.2.1.24), the enzyme which mediates the second step in heme biosynthesis. Aminotriazole decreases the incorporation of both δ-aminolevulinic acid-^{14}C and ^{59}Fe into microsomal heme and inhibits the stimulation of ^{59}Fe incorporation into microsomal heme produced by phenobarbital. Aminotriazole also inhibits the induction of rat hepatic microsomal cytochrome P-450 and that of the microsomal oxidations of ethylmorphine, norcodeine, and 3-methyl-4-monomethylaminoazobenzene produced by phenobarbital during the first 48 hr of treatment.

Since aminotriazole has no effect on the induction of the hepatic microsomal flavoprotein NADPH-cytochrome *c* reductase by phenobarbital, it appears to inhibit the biosynthesis of heme exclusive of an effect on protein synthesis. It is suggested that heme synthesis may be the controlling event in the synthesis of the microsomal hemoprotein cytochrome P-450, and that stimulation of heme synthesis represents one means by which phenobarbital produces an increase of cytochrome P-450 in hepatic microsomes.

INTRODUCTION

Substances, such as phenobarbital, which are known to stimulate hepatic microsomal drug oxidations and increase hepatic microsomal levels of cytochrome P-450 have been demonstrated to induce δ-aminolevulinic acid synthetase (1), the proposed initial and rate-limiting enzyme in the heme-biosynthetic pathway (1, 2). This suggests that phenobarbital may exert its stimulatory effect on drug metabolism by increasing the rate of heme synthesis, which in turn may regulate the synthesis of the hemoprotein cytochrome P-450. On the other hand, phenobarbital also induces hepatic microsomal NADPH-cytochrome *c* reductase (3, 4). This flavoprotein may control the rate of reduction of cytochrome P-450 and may also represent a possible rate-limiting step in the oxidation of certain drugs in hepatic microsomes (3, 5).

The herbicide 3-amino-1,2,4-triazole is known to inhibit hepatic δ-aminolevulinic acid dehydratase (EC 4.2.1.24), the enzyme mediating the second step of heme biosynthesis (6). Although inhibition of the second and presumably non-rate-limiting step seemed to be a rather inefficient method of inhibiting heme biosynthesis, the fact that aminotriazole could be employed in very high doses in the animal organism presented an opportunity of inhibiting the dehydratase to such an extent that it could assume a rate-controlling function.

Kato (7) has reported that the administration of aminotriazole to rats markedly

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decreased the activities of drug-metabolizing enzymes and the content of cytochrome P-450 in hepatic microsomes, but had no effect on the content of cytochrome b_5. Aminotriazole also inhibited the induction of cytochrome P-450 and of certain drug-metabolizing enzymes by phenobarbital. Kato concluded that these effects were due to inhibition of the biosynthesis of cytochrome P-450. However, no evidence of the effect of aminotriazole on heme synthesis was provided. Doses of the herbicide employed in that study were very high, and it is conceivable that they produced nonspecific effects, such as inhibition of protein synthesis. For example, inhibition of the synthesis of NADPH-cytochrome *c* reductase, a possible rate-limiting component of hepatic microsomal drug oxidations, could explain the effects of aminotriazole. The present report describes the effects of aminotriazole on the induction by phenobarbital of hepatic microsomal drug-metabolizing enzymes, cytochrome P-450, NADPH-cytochrome *c* reductase, and heme biosynthesis. A preliminary account of this work has been published (8).

EXPERIMENTAL PROCEDURE

Materials

Glucose 6-phosphate, yeast glucose 6-phosphate dehydrogenase, NADP, NADPH (type I), NADH (grade III), horse heart cytochrome *c* (type III), and δ-aminolevulinic acid hydrochloride were obtained from Sigma Chemical Company. δ-Aminolevulinic acid-4-^{14}C hydrochloride (specific activity, 37.4 mC/mmole), 3-amino-1,2,4-triazole-5-^{14}C (specific activity, 1.07 mC/mmole), and $^{59}FeCl_3$ were obtained from New England Nuclear Corporation. Ethylmorphine hydrochloride and norcodeine hydrochloride were obtained from Merck and Company, Inc. 3-Methyl-4-monomethylaminoazobenzene was a generous gift from Dr. James Miller of the McArdle Laboratory for Cancer Research of the University of Wisconsin. 3-Amino-1,2,4-triazole employed *in vitro* was generously supplied by the American Cyanamid Company, Agricultural Chemical Division, while that for studies *in vivo* was obtained from Eastman Organic Chemicals. All other agents were employed in the highest purity available.

Treatment of Animals

Male albino Holtzman rats weighing 130–170 g were used throughout these studies. Rats received intraperitoneal injections of phenobarbital (40 mg/kg) and/or aminotriazole (3 g/kg). Control rats received an equal volume of 0.9% NaCl. In all cases, except where stated otherwise, rats were fasted for 24 hr prior to death but were given water *ad libitum*.

Preparation of Microsomes

Studies on drug metabolism and spectral analyses. Rats were killed by decapitation; their livers were immediately excised, and 25% (w/v) homogenates were prepared in ice-cold 0.25 M sucrose containing 1 mM disodium EDTA, pH 7.4, in a manually operated homogenizer. Homogenates were centrifuged at 12,500 × *g* for 20 min at 0–4°. The postmitochondrial supernatant fractions were then centrifuged at 104,000 × *g* for 90 min in a Spinco model L ultracentrifuge, and the microsomal pellets were suspended in sucrose-EDTA such that each milliliter of the resulting suspension contained about 10 mg of protein.

Studies on incorporation of heme precursors into microsomal heme. For the measurement of the incorporation *in vivo* of precursors into microsomal heme, livers were first perfused with 80 ml of ice-cold 0.9% NaCl to remove hemoglobin. Homogenates were prepared in 1.15% KCl, and the postmitochondrial supernatant fractions were obtained by centrifugation at 9000 × *g* for 20 min at 0–4°. The microsomal pellets were prepared as described above, but were washed and suspended in 1.15% KCl such that each milliliter contained 15–20 mg of protein. The microsomal suspensions were free of hemoglobin as measured by the method of Nishibayashi and Sato (9).

Enzymatic Assays

Drug metabolism. The *N*-demethylation of ethylmorphine and the *O*-demethylation of norcodeine were determined by measuring the amount of formaldehyde formed by a modification of the method of Nash, as described previously (10). Ethylmorphine and norcodeine were employed in substrate concentrations of 8×10^{-4} and 9×10^{-4} M, respectively. The *N*-demethylation of 3-methyl-4-monomethylaminoazobenzene was determined by measuring the formation of formaldehyde using the chromotropic acid procedure (11). 3-Methyl-4-monomethylaminoazobenzene, dissolved in a small volume of acetone, was employed in a substrate concentration of 7×10^{-4} M. All observations were made during a time interval when reaction rates were linear.

NADPH-cytochrome c reductase. The activity of NADPH-cytochrome *c* reductase in microsomal preparations was measured on a Gilford model 2000 recording spectrophotometer as the rate of increase in absorbance at 550 mμ produced by the reduction of cytochrome *c*. Each cuvette of 1-cm light path contained, in a total volume of 3 ml, 1.0 μmole of sodium cyanide, 0.1 μmole of cytochrome *c*, 1 mg of microsomal protein, and 0.2 M potassium phosphate buffer, pH 7.4. At zero time, 0.4 μmole of NADPH was added to the sample cuvette to start the reaction. Enzymatic activity was determined by using 19.7 mM^{-1} cm^{-1} as the millimolar difference extinction coefficient at 550 mμ.

δ-Aminolevulinic acid dehydratase. The activity of δ-aminolevulinic acid dehydratase was determined by measuring the rate of porphobilinogen formation using a modification of the method of Gibson *et al.* (12). A postmitochondrial supernatant fraction prepared in 0.25 M sucrose was employed as the source of the enzyme. The reactions were carried out for 10 min at 38° without shaking in a Dubnoff metabolic incubator under an atmosphere of nitrogen to prevent oxidation of porphobilinogen. The reaction was terminated by the addition of 1 ml of a 10% trichloracetic acid solution containing 0.1 M mercuric chloride, and the precipitate was removed by centrifugation. The amount of porphobilinogen in the supernatant was estimated by the method of Mauzerall and Granick (13), using a modified Ehrlich reagent in 2 N perchloric acid. The apparent millimolar extinction coefficient at 555 mμ was assumed to be 61 mM^{-1} cm^{-1} (13).

Cytochrome P-450 determination. Cytochrome P-450 in microsomal preparations was estimated from the carbon monoxide difference spectra obtained by gassing dithionite-reduced samples with carbon monoxide for 1 min. Each cuvette of 1-cm light path contained a few milligrams of sodium dithionite and about 4 mg of microsomal protein per milliliter after dilution of the sucrose-EDTA microsomal suspension with 0.2 M potassium phosphate buffer, pH 7.4. Measurements were based on the differences in absorbance between 450 and 490 mμ.

Cytochrome b_5 determination. The cytochrome b_5 content in the microsomal preparations was estimated from the difference spectra between the oxidized and NADH-reduced forms in a Gilford model 2000 recording spectrophotometer. Each cuvette of 1-cm light path contained about 4 mg of microsomal protein per milliliter after dilution of the sucrose-EDTA microsomal suspension with 0.2 M phosphate buffer, pH 7.4. A millimolar extinction coefficient of 163 mM^{-1} cm^{-1} was employed for the difference in absorbance between 424 and 409 mμ (14).

Protein. Protein was determined by the biuret method (15).

Incorporation of Precursors into Microsomal Heme in Vivo

For the studies of heme synthesis *in vivo*, either δ-aminolevulinic acid-4-^{14}C hydrochloride or $^{59}FeCl_3$ was administered intraperitoneally to rats 1 or 4 hr, respectively, after the administration of the drugs. Livers were perfused and removed 60 min after injection of the precursor. Incorporation of precursors into microsomal heme was found to be linear throughout this period. When δ-aminolevulinic acid-^{14}C was used animals received a pulse-dose

of 10 μC. When $^{59}FeCl_3$ was employed, animals received a pulse-dose of 50 μC, and the radioactivity of the microsomal fractions was measured in a Packard Auto-Gamma spectrometer, series 5000. In these studies, rats were not fasted. The incorporation of precursors into microsomal heme was also determined in heme extracts from hepatic microsomes. One volume of microsomes was shaken for 15 min with 2 volumes of acetone containing 4% concentrated HCl. After brief centrifugation, the supernatant was removed and the heme was extracted into peroxide-free ether as described previously (16). In those instances when δ-aminolevulinic acid-^{14}C was employed, the radioactivity of the ether extracts was measured in a Packard Tri-Carb scintillation spectrometer after addition of 0.1 ml of the ether extract to 15 ml of the toluene scintillation fluid according to the method of Shuster and Jick (17). The efficiency for counting of ^{14}C in this system was 85%. Quenching was negligible. When $^{59}FeCl_3$ was employed, 10 ml of the ether extract were evaporated to dryness under nitrogen, 1 ml of ether was added, and the samples were counted as described above for the microsomal fractions.

The protoheme content of microsomes was measured by the method of Omura and Sato (18). The protoheme content of the ether extracts was determined by evaporating 2 ml of the ether extract to dryness under nitrogen, followed by the addition of 0.4 ml of pyridine, 0.1 ml of 2 N NaOH, and 2 ml of distilled water. The dithionite-reduced minus oxidized difference spectra were recorded on a Gilford model 2000 recording spectrophotometer in the same manner as for the microsomal preparations.

Measurement of Hexobarbital Metabolism in Vivo

To determine the metabolism of hexobarbital *in vivo*, sodium hexobarbital (82.5 mg/kg) was administered intraperitoneally. At selected time intervals after injection, the rats were killed and 1.5 ml of blood were drawn from the abdominal aorta into heparinized syringes. The hexobarbital content of the blood was determined by the method of Cooper and Brodie (19). Blood from rats that received only 0.9% NaCl was used to provide control values for the analysis.

RESULTS

Inhibition of hepatic δ-aminolevulinic acid dehydratase by aminotriazole in vitro. Table 1 shows the results of the effect of aminotriazole on rat hepatic δ-aminolevulinic acid dehydratase activity *in vitro*. Although aminotriazole inhibits this enzyme, it is not a potent inhibitor *in vitro* and the degree of inhibition depends on the concentration of δ-aminolevulinic acid. Studies are now in progress on the kinetic nature of this inhibition.

TABLE 1

Inhibition of hepatic δ-aminolevulinic acid dehydratase by aminotriazole in vitro

Reaction mixtures (3 ml) contained the postmitochondrial supernatant fraction equivalent to 250 mg of liver. A 10-min activation period was employed.

δ-Aminolevulinic acid	Aminotriazole	Porphobilinogen formed[a]	Inhibition
M	*M*	*mμmole/mg protein/min*	%
3×10^{-4}	0	0.055	
	5×10^{-2}	0.039	30.0
5×10^{-4}	0	0.062	
	5×10^{-2}	0.049	21.0
3×10^{-3}	0	0.076	
	5×10^{-2}	0.065	14.5

[a] Each value represents the mean of at least four experiments.

Effect of aminotriazole and phenobarbital on hepatic heme synthesis in vivo. Although aminotriazole appeared to be a rather weak inhibitor of rat hepatic δ-aminolevulinic acid dehydratase, the possibility still existed that treatment of rats with aminotriazole could produce inhibition of hepatic heme synthesis *in vivo*. The effects of treatment with aminotriazole on the incorporation of δ-aminolevulinic acid-4-^{14}C into microsomal heme *in vivo* are summarized in Table 2. From this table it can be seen that when aminotriazole is administered, a decrease of 40% in δ-aminolevulinic acid-^{14}C incorporation into extracted microsomal heme results. One hour after phenobarbital administration, the incorporation of δ-aminolevulinic acid-^{14}C into microsomal heme did not appear to be stimulated.

TABLE 2

Effects of aminotriazole and phenobarbital on incorporation of δ-aminolevulinic acid-4-^{14}C into microsomal heme in vivo

Rats received intraperitoneally a 60-min pulse-dose of 10 μC of δ-aminolevulinic acid-^{14}C 1 hr after the intraperitoneal administration of aminotriazole (3 g/kg) and/or phenobarbital (40 mg/kg). Control animals received an equal volume of 0.9% NaCl 1 hr before the pulse-dose. Incorporation into extracted microsomal heme was determined as described in the text. The control value was 8729 cpm/mμmole of protoheme per milliliter of ether extract.

Treatment	Percent of control[a]
Aminotriazole	59.4 ± 9.1
Phenobarbital	87.0 ± 10.7
Both agents	77.2 ± 20.0

[a] Values represent the mean ± standard error of three experiments.

Phenobarbital administration results in an increased level of cytochrome P-450 in hepatic microsomes. One explanation of this increase may be a stimulation of hepatic heme synthesis, which would be subject to inhibition by aminotriazole. The effects of aminotriazole and phenobarbital on the incorporation of $^{59}FeCl_3$ into hepatic microsomal heme *in vivo* are compared in Table 3. Four hours after administration, phenobarbital produced a 45–55% stimulation of ^{59}Fe incorporation into microsomal heme, which was inhibited by aminotriazole. When aminotriazole alone was administered, a 40–50% decrease in incorporation of ^{59}Fe was observed.

TABLE 3

Effects of aminotriazole and phenobarbital on incorporation of $^{59}FeCl_3$ into hepatic microsomal heme in vivo

Rats received intraperitoneally a 60-min pulse-dose of 50 μC of $^{59}FeCl_3$ 4 hr after the intraperitoneal administration of aminotriazole (3 g/kg) and/or phenobarbital (40 mg/kg). Control animals received an equal volume of 0.9% NaCl 4 hr before the pulse-dose. Incorporation was determined as described in the text. The control value for microsomes was 238.4 cpm/mμmole of protoheme per milliliter of microsomes, while that for extracted heme was 5.3 cpm/mμmole of protoheme per milliliter of ether extract.

	Percent of control[a]	
Treatment	Microsomes	Extracted microsomal heme
Aminotriazole	62.7 ± 16.7	54.8 ± 11.9
Phenobarbital	155.5 ± 14.5	144.7 ± 21.5
Both agents	79.4 ± 19.6	69.6 ± 12.7

[a] Values represent the mean ± standard error of three experiments.

Effect of aminotriazole on induction of hepatic microsomal cytochrome by phenobarbital. If phenobarbital increases cytochrome P-450 levels by stimulating heme synthesis, aminotriazole should inhibit the induction of cytochrome P-450 produced by phenobarbital. Figure 1 shows the effect of aminotriazole on the induction of cytochrome P-450 in hepatic microsomes produced by phenobarbital. Phenobarbital administration results in significant increases in cytochrome P-450 ($p < 0.05$) levels after 8 hr, and these increases are considerably diminished by the simultaneous administration of aminotriazole. Initial studies showed that aminotriazole began to lose its effect after about 48 hr. Treatment with aminotriazole alone produces a maximum decrease in cytochrome P-450 levels of nearly 50% approximately 16 hr after administration. Kato (7) ob-

TABLE 4

Effects of aminotriazole and phenobarbital on hepatic microsomal cytochrome b_5 levels

Rats received intraperitoneal injections of aminotriazole (3 g/kg) and/or phenobarbital (40 mg/kg) at zero time. Control animals received an equal volume of 0.9% NaCl. Rats either were killed 8, 16, or 24 hr later or received another series of injections 24 hr after the first, and were then killed 24 hr later. Cytochrome b_5 determinations were made as described in the text.

Treatment for	Cytochrome b_5 levels[a]			
	Control	Aminotriazole	Phenobarbital	Both agents
hr	*mμmole/mg protein*			
8	0.138 ± 0.044	0.059 ± 0.018	0.089 ± 0.004	0.092 ± 0.049
16	0.088 ± 0.007	0.095 ± 0.011	0.113 ± 0.016	0.110 ± 0.007
24	0.090 ± 0.016	0.096 ± 0.016	0.109 ± 0.013	0.123 ± 0.019
48	0.079 ± 0.022	0.095 ± 0.012	0.137 ± 0.014	0.119 ± 0.010

[a] Values represent the mean ± standard error of at least four experiments. No significant differences were observed when cytochrome b_5 values in microsomes from animals treated with aminotriazole and/or phenobarbital were compared with the values obtained in microsomes from control animals.

served a 50% decrease in microsomal cytochrome P-450 content at doses of aminotriazole similar to those employed in the current study.

It is interesting that cytochrome b_5 levels were not affected by either aminotriazole or phenobarbital during the time periods studied (Table 4). These findings are in agreement with those of Kato (7). That phenobarbital does not increase cytochrome b_5 levels has been reported by Orrenius and Ernster (3, 4).

Effect of aminotriazole on induction of

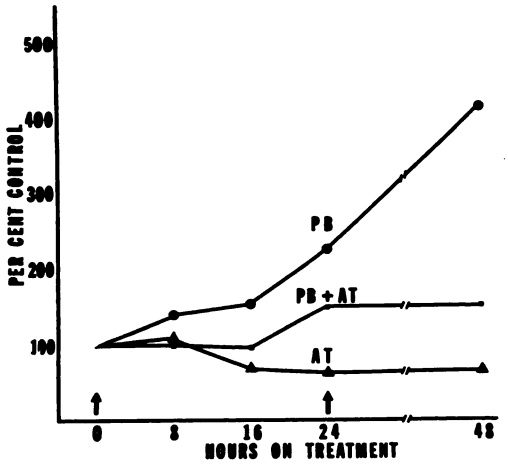

FIG. 1. *Effect of aminotriazole (3 mg/kg) (AT) on the induction of hepatic microsomal cytochrome P-450 by phenobarbital (40 mg/kg) (PB)*

Drugs were administered intraperitoneally at zero time, control animals receiving an equal volume of 0.9% NaCl, and the animals either were killed 8, 16, or 24 hr later or received a second series of injections 24 hr after the first and then were killed 24 hr later. Cytochrome P-450 was determined as described in the text. Each point represents the mean of at least four experiments. The average control value was 0.023 $\Delta A_{450-490}$ unit/mg of protein.

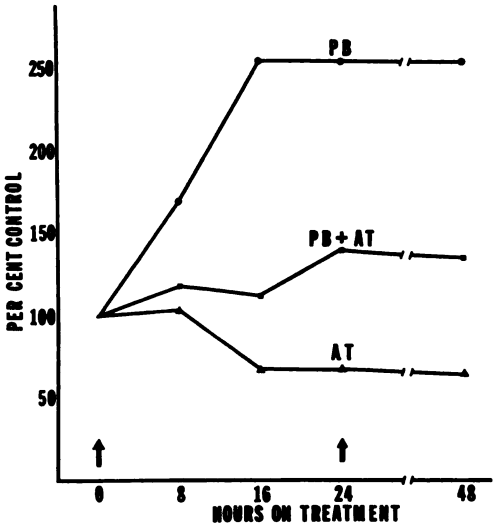

FIG. 2. *Effect of aminotriazole (3 g/kg) (AT) on induction of the N-demethylation of ethylmorphine by phenobarbital (40 mg/kg) (PB)*

Conditions were the same as described for Fig. 1. Each point represents the mean of at least four experiments. The average control value was 2.76 mμmoles of formaldehyde per milligram of protein per minute.

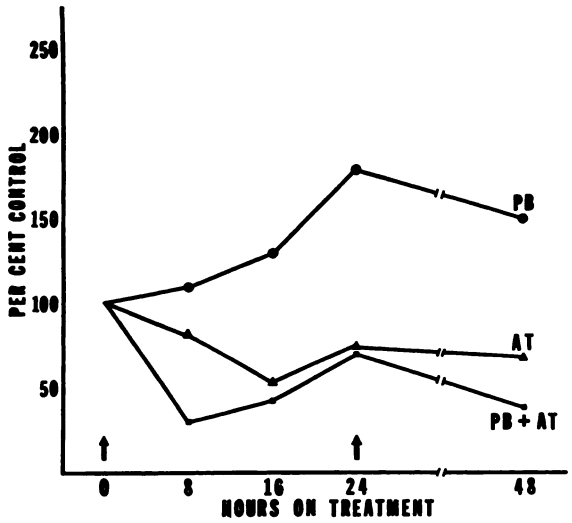

FIG. 3. *Effect of aminotriazole (3 mg/kg) (AT) on induction of the O-demethylation of norcodeine by phenobarbital (40 mg/kg) (PB)*

Conditions were the same as described for Fig. 1. Each point represents the mean of at least four experiments. The average control value was 0.74 mμmole of formaldehyde per milligram of protein per minute.

ethylmorphine, norcodeine, and 3-methyl-4-monomethylaminoazobenzene demethylation by phenobarbital. Figure 2 shows that treatment of rats with phenobarbital results in induction of the *N*-demethylation of ethylmorphine. This action of phenobarbital is inhibited by aminotriazole. Treatment of rats with aminotriazole alone produced a maximum decrease of nearly 40% in ethylmorphine demethylation approximately 16 hr after administration. Phenobarbital administration also results in induction of the *O*-demethylation of norcodeine, although the time course of induction and the degree of stimulation of norcodeine *O*-demethylation differ from those of the *N*-demethylation of ethylmorphine (Fig. 3). After an initial stimulation of norcodeine *O*-demethylation during the first 24 hr of treatment, the metabolic activity begins to fall to normal levels. It has been shown that after 4 days of phenobarbital treatment, norcodeine *O*-demethylation is not significantly increased over control values while there is a 3–4-fold increase in ethylmorphine *N*-demethylation (10). Another difference between the induction of ethylmorphine and norcodeine oxidation by phenobarbital is that not only does aminotriazole reverse the phenobarbital-induced increase in norcodeine *O*-demethylation, but, after the combination of aminotriazole and phenobarbital, norcodeine *O*-demethylation is significantly ($p < 0.05$) depressed to a greater extent than by aminotriazole alone (Fig. 3).

The administration of phenobarbital also results in induction of the *N*-demethylation of 3-methyl-4-monomethylaminoazobenzene, which is prevented by aminotriazole (Fig. 4). The time course of 3-methyl-4-monomethylaminoazobenzene induction resembles that seen with norcodeine, although other studies show that *N*-demethylation of 3-methyl-4-monomethylaminoazobenzene remains induced after 4 days of phenobarbital treatment (20) whereas *O*-demethylation of norcodeine does not (10). Although these stimulation patterns seem different, when stimulation occurs, aminotriazole effects an inhibition. After about 48 hr, however, aminotriazole loses its inhibitory effect on phenobarbital stimulation of drug metabolism.

Effect of aminotriazole on induction of hepatic microsomal NADPH-cytochrome c reductase by phenobarbital. NADPH-cytochrome *c* reductase is a microsomal

non-heme flavoprotein which has been implicated as a component of the hepatic microsomal electron transport system (3, 21) and is induced by phenobarbital (3, 4). As shown in Fig. 5, phenobarbital treatment results in the induction of this enzyme. However, aminotriazole does not inhibit this induction, nor does it alter the level of activity of the enzyme in microsomes derived from control rats. Several experiments were performed in which the incorporation of uniformly labeled L-leucine-^{14}C into microsomal protein *in vitro* was determined. When the incorporation of the labeled amino acid into microsomal protein was determined using a microsomal preparation derived from an aminotriazole-treated animal, no inhibitory effect was observed. These data indicate that aminotriazole is not a general inhibitor of protein synthesis and that NADPH-cytochrome *c* reductase does not control the rate of metabolism of the substrates used in these studies.

Effect of aminotriazole on hepatic microsomal drug metabolism in vitro and in vivo. Because of the high dose of aminotriazole administered *in vivo*, it seemed quite possible that this substance might be present in microsomal preparations at sufficient concentrations to exert a direct inhibition on drug oxidations. Indeed, the addition of high concentrations (10^{-2} M) of aminotriazole to microsomal preparations results in a type II spectral change (22). Since this is characteristic of substrate interaction with cytochrome P-450, aminotriazole might inhibit the metabolism of the substrates employed by competing with them for cytochrome P-450. Studies were performed in which aminotriazole was added to reaction mixtures containing the drug substrates and microsomes from livers of untreated rats. Table 5 shows that concentrations of aminotriazole greater than 10^{-3} M were required to produce significant inhibition of the substrates employed in these studies. The concentration of aminotriazole required to produce 50% inhibition (I_{50}) of the *N*-demethylation of 3-methyl-4-monomethylaminoazobenzene is about 10 times greater than that required for the demethylation of either ethylmorphine or norcodeine. When microsomes obtained

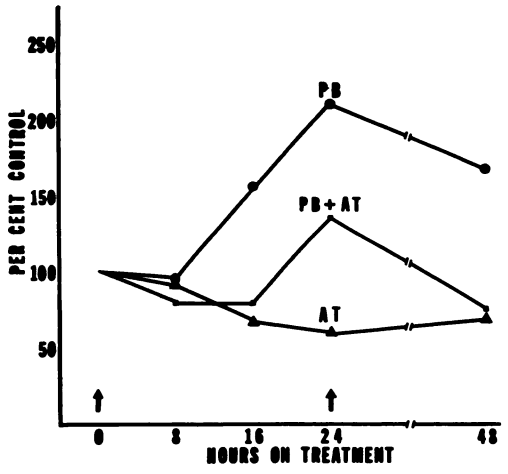

FIG. 4. *Effect of aminotriazole (3 mg/kg) (AT) on induction of the N-demethylation of 3-methyl-4-monomethylaminoazobenzene by phenobarbital (40 mg/kg) (PB)*

Conditions were the same as described for Fig 1. Each point represents the mean of at least four experiments. The average control value was 0.58 mμmole of formaldehyde per milligram of protein per minute.

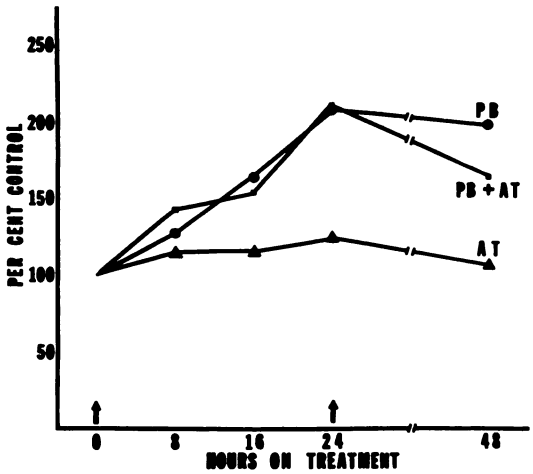

FIG. 5. *Effect of aminotriazole (3 mg/kg) (AT) on the induction of NADPH-cytochrome c reductase by phenobarbital (40 mg/kg) (PB)*

Conditions were the same as described for Fig. 1. Each point represents the mean of at least four experiments. The average control value was 39.1 mμmoles of cytochrome *c* reduced per milligram of protein per minute.

TABLE 5

Effect of aminotriazole on hepatic microsomal drug metabolism in vitro

Metabolism of the drug substrates was determined as described in the text, employing microsomal preparations from untreated rats. Control reactions contained no aminotriazole. Control values (nanomoles of formaldehyde per milligram of protein per minute) were 2.19 for ethylmorphine *N*-demethylation, 0.71 for norcodeine *O*-demethylation, and 0.59 for 3-methyl-4-monomethylaminoazobenzene *N*-demethylation.

Aminotriazole concentration	Percent of control[a]		
	Ethylmorphine	Norcodeine	3-Methyl-4-monomethylaminoazobenzene
M			
10^{-3}	90.0	83.2	94.6
5×10^{-3}	72.9	76.5	81.5
10^{-2}	69.1	66.7	76.3
5×10^{-2}	33.6	42.7	61.6
10^{-1}	22.0	24.0	56.5
I_{50}[b]	2.2×10^{-2} M	3.1×10^{-2} M	2.1×10^{-1} M

[a] Each value represents the mean of three experiments.

[b] Concentration of aminotriazole at which 50% inhibition of demethylation occurred.

from the livers of rats which had been treated with phenobarbital for 48 hr were employed, identical results were obtained. This indicates that the induced enzymes have the same degree of sensitivity toward aminotriazole as do the enzymes present in control livers.

When the content of aminotriazole in microsomal preparations derived from aminotriazole-treated rats was determined chemically by means of a diazotization reaction with *N*-naphthylethylenediamine hydrochloride (23), concentrations in the micromolar range were found. However, this did not rule out binding of aminotriazole to microsomes, which might pos-

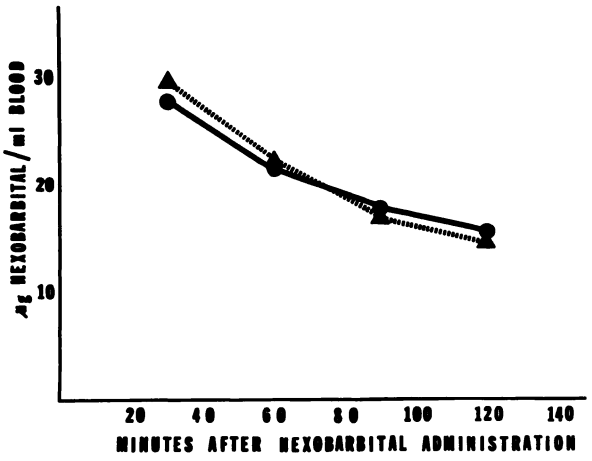

FIG. 6. *Effect of aminotriazole on hexobarbital metabolism in vivo*

Rats received intraperitoneal injections of either aminotriazole (3 mg/kg) or an equal volume of 0.9% NaCl. Sodium hexobarbital (82.5 mg/kg) was administered intraperitoneally 24 hr later. Blood was taken from the animals at the time intervals indicated and assayed for hexobarbital as described in the text. ●, Values obtained from control animals; ▲, values obtained from aminotriazole-treated animals. Each point represents the mean of values obtained from at least two rats.

sibly result in a direct inhibitory effect. Rats were treated with 3-amino-1,2,4-triazole-5-^{14}C (3 g/kg, 0.2 mC/kg) for the same time intervals used throughout these studies, and concentrations were determined to be between 2 and 5×10^{-3} M. This could be explained by the presence of metabolites of aminotriazole. However, the high concentration of label may also be explained by the incorporation of aminotriazole into microsomal protein after conversion to 3-amino-1,2,4-triazolylalanine. This does occur in *Escherichia coli* (24). However, these concentrations are about 10 times lower than the apparent I_{50} values obtained for aminotriazole (Table 5).

Disappearance of hexobarbital is often used as a measure of microsomal drug metabolism *in vivo* (19). Figure 6 shows the effect of treatment with aminotriazole on the metabolism of hexobarbital *in vivo*. At 24 hr after administration of aminotriazole no alteration in the biological half-life of hexobarbital was observed. These data suggest that the concentration of aminotriazole in rat liver is insufficient to produce direct inhibition of drug metabolism. Kato has reported that aminotriazole inhibits the metabolism of pentobarbital in female rats *in vivo* (7).

DISCUSSION

These studies establish the effect of aminotriazole on heme synthesis, on the induction of cytochrome P-450, and on the induction of microsomal oxidases produced by the administration of phenobarbital. Initial studies showed that aminotriazole inhibited δ-aminolevulinic acid dehydratase in a soluble fraction from rat liver, confirming the earlier observation of Tschudy and Collins in mice (6). Although aminotriazole is a rather weak inhibitor of the enzyme, sufficiently high doses could be administered acutely to produce inhibition of heme synthesis. The incorporation of δ-aminolevulinic acid-^{14}C into microsomal heme was decreased almost 40% from control values 1 hr after the administration of aminotriazole to rats. Four hours after the administration of phenobarbital, the incorporation of ^{59}Fe into microsomal heme was about 50% greater than the control. This stimulatory action of phenobarbital on ^{59}Fe incorporation was completely prevented when aminotriazole was administered together with phenobarbital. The incorporation of ^{59}Fe into microsomal heme was decreased to nearly 60% of the control value 4 hr after the administration of aminotriazole. This compound also prevented the phenobarbital-induced increase in hepatic microsomal cytochrome P-450 levels. This suggests that one possible mechanism for the induction of cytochrome P-450 by phenobarbital is the stimulation of heme synthesis. However, no good correlation was found between the rate of increase of cytochrome P-450 and the oxidations of ethylmorphine, norcodeine, and 3-methyl-4-monomethylaminoazobenzene. This may mean that a certain amount of the newly synthesized cytochrome P-450 is not functional in drug metabolism (5). The increase in heme synthesis after phenobarbital administration has been postulated to be due to the induction of the rate-limiting enzyme in heme biosynthesis, δ-aminolevulinic acid synthetase (1). Thus, the loss of inhibition by aminotriazole after 48 hr may be due to an increased endogenous level of δ-aminolevulinic acid generated by increased amounts of hepatic δ-aminolevulinic acid synthetase induced by phenobarbital. The degree of inhibition of δ-aminolevulinic acid dehydratase was shown to be dependent on substrate concentration. These findings also suggest that heme synthesis may control the rate of cytochrome P-450 synthesis much as it may control hemoglobin synthesis in reticulocytes (25–27). Many of our observations on the effect of aminotriazole on drug-metabolizing enzymes, cytochrome P-450, and cytochrome b_5 are in agreement with previous findings by Kato (7).

It is interesting that aminotriazole did not affect the induction of NADPH-cytochrome *c* reductase by phenobarbital. Therefore, aminotriazole did not appear to alter protein synthesis in these studies. Furthermore, since stimulation of drug metabolism was inhibited, the role of NADPH-cytochrome *c* reductase as the

rate-limiting component in drug metabolism may be questioned if it is responsible for the reduction of cytochrome P-450. It is possible, however, that the reduction of cytochrome P-450 may not be mediated by NADPH-cytochrome *c* reductase.

Although the dose of aminotriazole (3 g/kg) employed *in vivo* throughout this study was relatively high, only a small percentage of the effects could be due to a direct inhibitory action of this agent on the hepatic drug-metabolizing system. Aminotriazole had no effect on the biological half-life of hexobarbital when hexobarbital metabolism was studied *in vivo* 24 hr after aminotriazole administration. The determination of free aminotriazole in microsomal preparations from livers of aminotriazole-treated rats showed levels far below those required for significant direct inhibition of drug metabolism. These data conflict with those of Kato (7), who concluded that aminotriazole (2 g/kg) decreased the metabolism of pentobarbital *in vivo*. However, Kato did not compare the rate of disappearance of pentobarbital in control and aminotriazole-treated animals, but determined levels of pentobarbital in serum and brain at various time periods after aminotriazole administration. Indeed, he showed elevated levels of pentobarbital 2 hr after administration of aminotriazole, a time when its effects on microsomal hemoproteins are not yet revealed. Therefore, Kato may have been observing the direct effect of aminotriazole on pentobarbital metabolism. His studies were performed in female rats and may also reflect a sex difference in the response to aminotriazole.

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