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The Effect of 3-Amino-1,2,4-triazole on the Phenobarbital-Induced Formation of Hepatic Microsomal Membranes

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SUMMARY

3-Amino-1,2,4-triazole was administered to rats in combination with phenobarbital. The hepatic endoplasmic reticulum was studied chemically and by electron microscopy. The increases in cytochrome P-450 and drug hydroxylase activity produced by phenobarbital alone were partially counteracted by administration of aminotriazole together with phenobarbital. The increases in membranes of the endoplasmic reticulum, observed by electron microscopy and measured by microsomal phospholipid, were comparable in animals receiving phenobarbital alone and in combination with aminotriazole. Aminotriazole treatment alone does not produce proliferation of endoplasmic reticular membranes. In the presence of an inducer such as phenobarbital, interference with cytochrome P-450 biosynthesis by aminotriazole does not affect induction of the formation of membranes of the hepatic endoplasmic reticulum. These results suggest that induced increases of cytochrome P-450 and of the membranes of endoplasmic reticulum may be controlled by separate mechanisms.

INTRODUCTION

The increases in hepatic endoplasmic reticulum and in drug-metabolizing activity following phenobarbital treatment are regarded as adaptive responses to the drug (1). The herbicide 3-amino-1,2,4-triazole inhibits heme biosynthesis (2). Aminotriazole administered together with phenobarbital for 1 and 2 days partially counteracted the increases in drug-metabolizing activity and in cytochrome P-450 levels produced by phenobarbital given alone (3, 4).

Membranes of the endoplasmic reticulum reticulum are maximally increased after 4 days of phenobarbital treatment (5). Does interference with induction of cytochrome P-450 biosynthesis affect the formation of membranes of the endoplasmic reticulum? The following study was designed to answer this question.

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MATERIALS AND METHODS

Treatment of animals. Male Sprague-Dawley rats weighing approximately 150 g received both sodium phenobarbital dissolved in sterile water, 50 mg/kg, and aminotriazole (Aldrich Chemical Company), 3 g/kg, dissolved in 0.9% NaCl. Animals were given these compounds intraperitoneally, at

the same time at separate injection sites, once daily for 4 days. Three other groups of animals served as controls. One group received 0.9% NaCl only, the second group received phenobarbital alone, and the third group received aminotriazole alone.

The NaCl and phenobarbital control animals were pair-fed with the experimental groups on laboratory chow (Rockland mouse/rat diet, Teklad, Inc.). The animals receiving aminotriazole alone ate less than the other groups and were not pair-fed.

For the duration of the experiment, the animals were placed in individual cages in an environment in which light, temperature, and humidity were automatically controlled.

On the fifth day, approximately 24 hr after the last injection, the animals were killed by a blow to the head. Livers were perfused with approximately 60 ml of ice-cold 0.25 M sucrose into the portal vein. Specimens for electron microscopy were obtained prior to perfusion.

Enzymatic assays. All enzymatic determinations were done on unfractionated 10% (w/v) liver homogenates from individual animals, prepared in 0.25 M sucrose–0.01 M Tris-HCl buffer, pH 7.8. Benzpyrene hydroxylase activity was assayed by the procedure of Kuntzman et al (6). Aniline hydroxylase activity was determined by the method of Brodie and Axelrod (7).

For the measurement of enzyme activities, an NADPH-generating system was added to each sample in excess. It consisted of 54 μmoles of glucose 6-phosphate, 4 units of glucose 6-phosphate dehydrogenase (from yeast, analytical reagent grade, Boehringer-Mannheim), 3.8 μmoles of NADP, and 10 μmoles of $MgCl_2$ in 0.1 M potassium phosphate buffer, pH 7.4. Aniline phosphate was added in substrate concentration of 1.3 μmoles in a final volume of 5 ml. 3,4-Benzpyrene (0.2 μmole dissolved in 0.1 ml of acetone) was added to a final volume of 3.0 ml. Nicotinamide was omitted from the assay because it has been shown to inhibit the aniline hydroxylase and aminopyrine demethylase reactions (8).

Preparation of microsomes. A 10% (w/v) homogenate of liver was prepared in 0.25 M sucrose–0.01 M Tris-HCl buffer, pH 7.8, and centrifuged at 9500 × *g* for 25 min at 0–4° in a Spinco model L ultracentrifuge. The supernatant fluid was centrifuged at 105,000 × *g* for 60 min. The pellets were resuspended and centrifuged again for 60 min at 105,000 × *g*. The resulting pellet was resuspended in 1.15% KCl.

Cytochrome P-450. This was measured as

TABLE 1

Effect of aminotriazole on induction of microsomal protein

The mean results ± standard deviation are represented. The increase in microsomal protein content was not significantly different in the animals treated with phenobarbital and aminotriazole in comparison to those receiving phenobarbital alone. The animals treated with aminotriazole alone lost more body weight than did the other groups. Aminotriazole treatment alone compared to NaCl controls does not significantly increase microsomal protein.

Treatment	Perfused livers			Unperfused livers		
	No. of animals	Liver to body weight ratio	Total microsomal protein[a]	No. of animals	Liver to body weight ratio	Total microsomal protein[a]
		%			%	
0.9% NaCl	8	4.45 ± 0.44[b]	93 ± 11[b]	7	3.71 ± 0.40	83 ± 10[b]
Phenobarbital	8	5.58 ± 0.48	147 ± 26[c]	7	4.66 ± 0.32[b]	158 ± 45[c]
Phenobarbital + aminotriazole	17	6.27 ± 0.55	133 ± 23[c]	12	6.06 ± 0.60	144 ± 27[c]
Aminotriazole	8	4.74 ± 0.50[b]	99 ± 16[b]	6	4.69 ± 0.44[b]	98 ± 16[b]

[a] Values are expressed as milligrams per gram of liver × [(liver weight/body weight) × 100].

[b] Differences between means in the same column, designated by the same superscript, are not statistically significant. Other differences are significant ($p < 0.05$).

[c] Differences between means in the same column, designated by the same superscript, are not statistically significant. Other differences are significant ($p < 0.05$).

described elsewhere (9), except that CO was bubbled through the sample for 60 sec.

Microsomal protein. Microsomal protein was measured by the method of Lowry *et al.* (10).

Microsomal phospholipid. Lipids were extracted by the method of Folch, Lees, and Sloane Stanley (11). The phosphorus was determined on an AutoAnalyzer by the method of Fiske and SubbaRow (12).

Preparation of tissue for electron microscopy. Small samples were fixed in cold 1% osmic acid in 2.67% *s*-collidine buffer. They were dehydrated with alcohols and embedded in Epon 812 (13). Sections were cut with LKB Ultrotome III, employing a diamond knife, and stained with lead citrate. They were studied with a Hitachi HS-7S electron microscope, after light microscopic localization in the liver lobule, using 1-μ sections stained with periodic acid–Schiff and toluidine blue (14). Periportal and midzonal regions were selected for electron microscopy, since the endoplasmic reticulum levels in these regions are normally the lowest (15) and the response to induction is therefore more pronounced.

TABLE 2

Effect of aminotriazole on induction of benzpyrene hydroxylation

The mean results ± standard deviation are represented. Phenobarbital treatment raises benzpyrene hydroxylase activity almost to 3 times and cytochrome P-450 levels to 5 times NaCl control values. With phenobarbital and aminotriazole the increase in benzpyrene hydroxylase activity is prevented, and the values are similar to NaCl controls. In contrast to the effect of phenobarbital and aminotriazole on benzpyrene hydroxylase activity, total cytochrome P-450 has increased to 1.7 times the control values. When compared to phenobarbital treatment alone, the induced increase of cytochrome P-450 is diminished. After aminotriazole treatment alone, benzpyrene hydroxylase activity is reduced to approximately 30% and total cytochrome P-450 is reduced to approximately 70% of NaCl-treated controls ($p < 0.05$).

Treatment	No. of animals	Total hydroxybenzpyrene[a]	Total cytochrome P-450[b]
0.9% NaCl	8	183 ± 60[c]	74 ± 17
Phenobarbital	8	530 ± 194	372 ± 100
Phenobarbital + aminotriazole	17	215 ± 124[c]	131 ± 43
Aminotriazole	8	59 ± 19	51 ± 16

[a] Values are expressed as micrograms per gram of liver × [(liver weight/body weight) × 100].

[b] Values are expressed as nanomoles per gram of liver × [(liver weight/body weight) × 100].

[c] Differences between means designated by the same superscript are not statistically significant. Other differences are significant ($p < 0.05$).

TABLE 3

Effect of aminotriazole on induction of aniline hydroxylation

The mean results ± standard deviation are represented. Phenobarbital treatment increases aniline hydroxylase activity 2.6 times and cytochrome P-450 levels to 5.6 times control values. Treatment with phenobarbital and aminotriazole diminishes this effect, but aniline hydroxylase activity and cytochrome P-450 levels are increased 1.5 times and 2.2 times, respectively, above NaCl controls. When values from animals treated with aminotriazole alone are compared with NaCl controls, the small increase in aniline hydroxylase activity and the small decrease in cytochrome P-450 levels are not significant.

Treatment	No. of animals	Total *p*-aminophenol[a]	Total cytochrome P-450[b]
0.9% NaCl	7	1.01 ± 0.24[c]	56 ± 10[c]
Phenobarbital	7	2.64 ± 0.50	295 ± 94
Phenobarbital + aminotriazole	12	1.58 ± 0.65[d]	122 ± 38
Aminotriazole	6	1.12 ± 0.33[c, d]	44 ± 15[c]

[a] Values are expressed as micromoles per gram of liver × [(liver weight/body weight) × 100].

[b] Values are expressed as nanomoles per gram of liver × [(liver weight/body weight) × 100].

[c] Differences between means in the same column, designated by the same superscript, are not statistically significant. Other differences are significant ($p < 0.05$).

[d] Differences between means in the same column, designated by the same superscript, are not statistically significant. Other differences are significant ($p < 0.05$).

RESULTS

The effects of phenobarbital and aminotriazole, alone and in combination, on liver weight and microsomal protein content are

shown in Table 1. The liver weight to body weight ratios in the groups of rats that received phenobarbital alone or together with aminotriazole were increased, as were the total microsomal protein contents. Perfusion of the livers with 0.25 M sucrose increased the apparent liver to body weight ratios, but did not affect the total microsomal yield.

Microsomal hydroxylations. Increased benzpyrene hydroxylase activity, which was obtained after phenobarbital treatment, was not found after administration of phenobarbital and aminotriazole (Table 2).

Another microsomal substrate, ethylmorphine, was tested for comparison, using isolated microsomes, and the demethylation of this compound closely followed the results obtained with benzpyrene.

The increase in hydroxylation of aniline, as seen with phenobarbital alone, was partially counteracted by phenobarbital together with aminotriazole (Table 3).

Phenobarbital treatment compared to NaCl controls increased benzpyrene hydroxylase activity 3-fold and aniline hydroxylase activity 2.5-fold, while increasing cytochrome P-450 levels 5-fold. In compari-

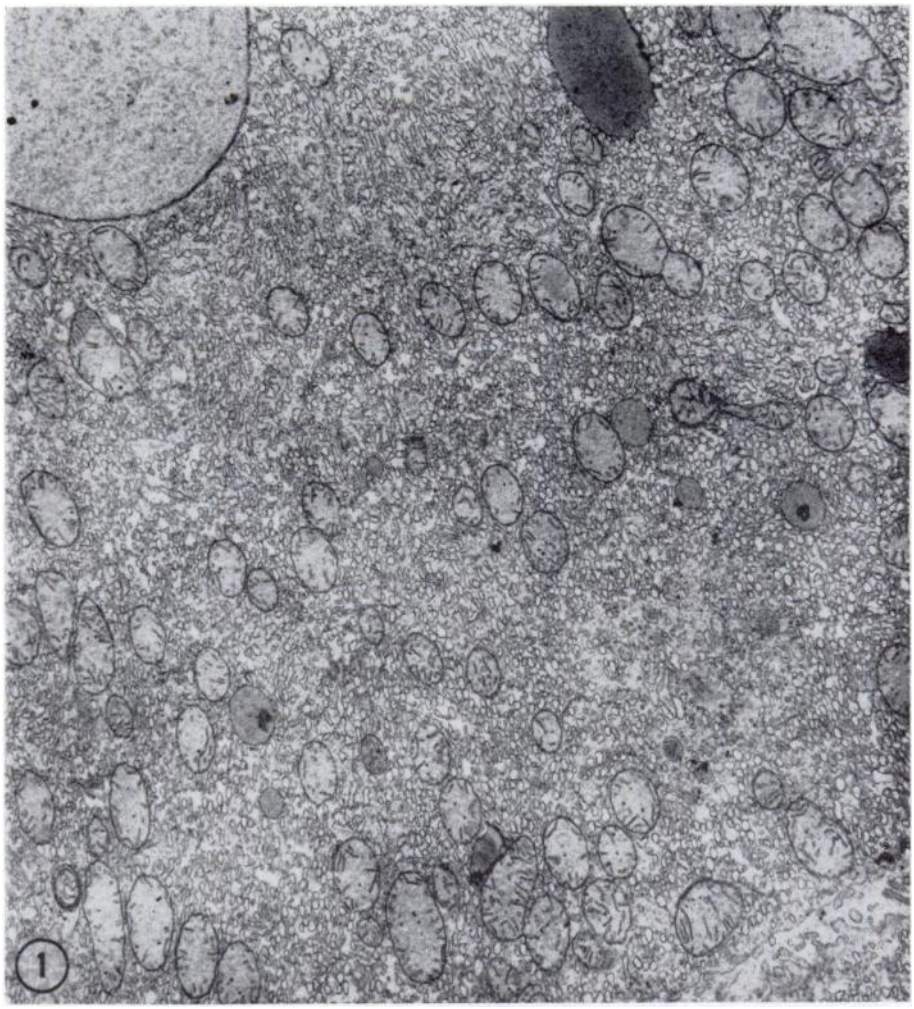

FIG. 1. *Hepatocyte from rat treated with phenobarbital alone*
Extensive increase of the smooth endoplasmic reticulum and depletion of glycogen are apparent. ×7500.

son to NaCl controls, the administration of phenobarbital together with aminotriazole did not significantly increase benzpyrene hydroxylase activity, while aniline hydroxylase activity increased 1.5-fold and cytochrome P-450 levels increased approximately 2-fold.

Morphology. Electron microscopic examination of hepatocytes after phenobarbital treatment alone revealed normal mitochondria, a loss of glycogen, and prominently increased smooth endoplasmic reticulum (Fig. 1). In animals receiving only aminotriazole, smooth and rough endoplasmic reticulum appeared normal (Fig. 2); the mitochondria varied in size and shape, and focal loss of outer mitochondrial membranes and focal swelling of the mitochondrial matrix were observed (Fig. 3).

Treatment with both phenobarbital and aminotriazole resulted in a loss of glycogen (Fig. 4). The smooth endoplasmic reticulum was increased in amount and distribution, with slight vesicle formation, but this was less pronounced than with phenobarbital alone. Rough endoplasmic reticulum content was diminished. The mitochondria displayed the same variation in size and shape as with aminotriazole alone, as well as focal loss of

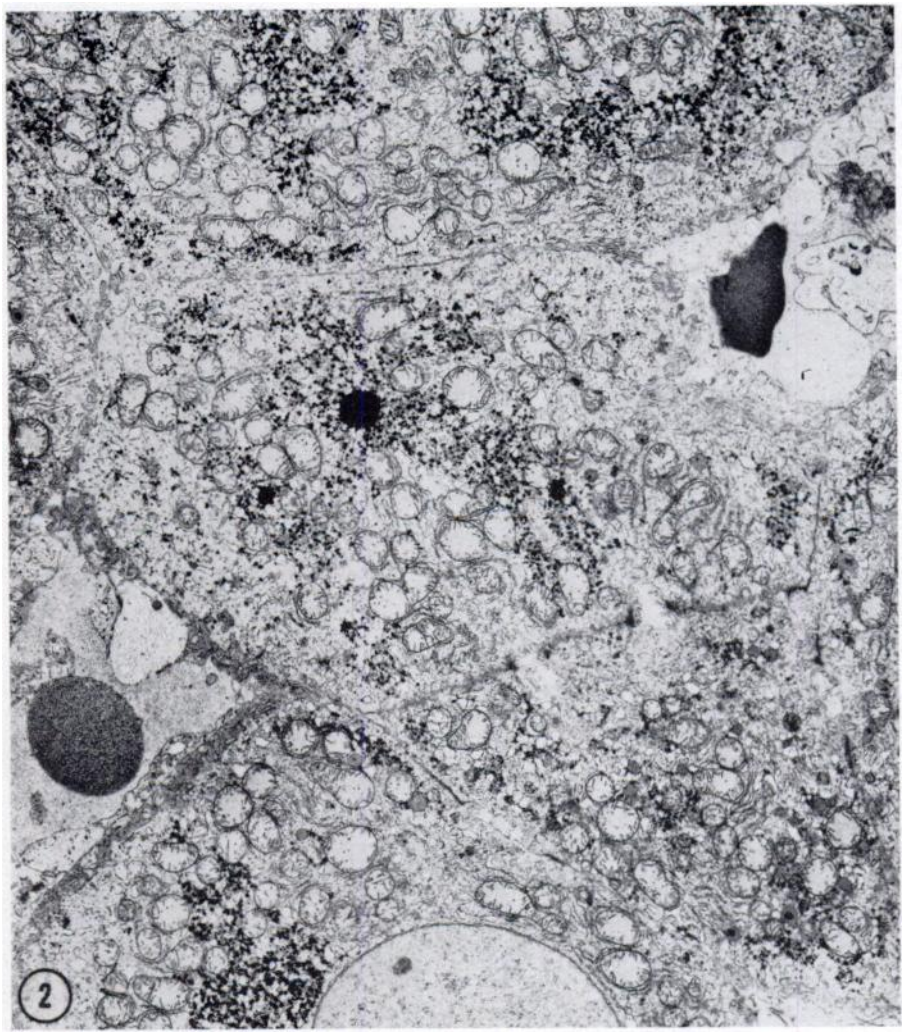

FIG. 2. *Hepatocyte from rat treated with aminotriazole alone*
Glycogen is preserved and smooth endoplasmic reticulum is not increased. × 5000.

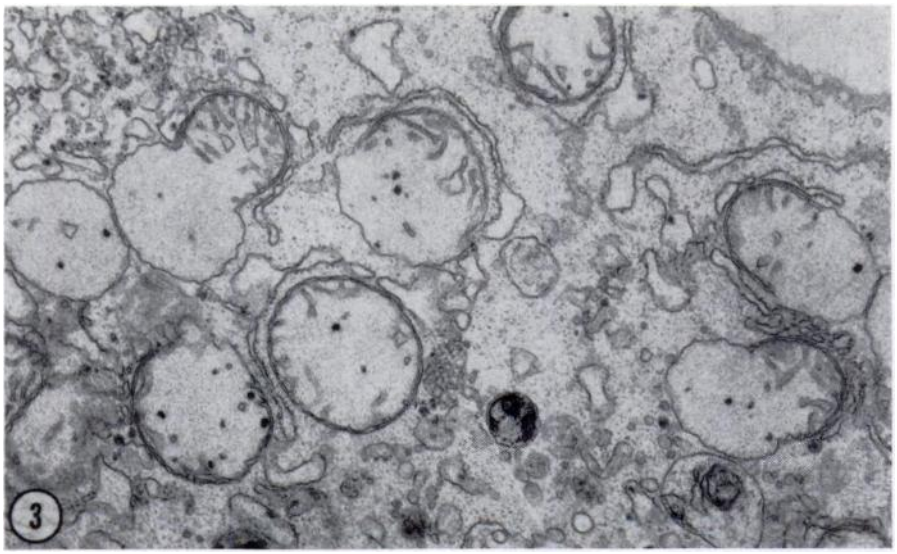

FIG. 3. *Mitochondria in hepatocyte from rat treated with aminotriazole alone*
Focal absence of outer membranes, with focal swelling of the mitochondrial matrix, has occurred. × 30,000.

TABLE 4

Effect of aminotriazole on induction of microsomal phospholipid

The mean results ± standard deviation are represented. Phenobarbital treatment increases microsomal phospholipid nearly 2 times control values. The increase in microsomal phospholipid following phenobarbital and aminotriazole is similar to that seen following phenobarbital treatment alone. Microsomal phospholipid is similar in NaCl controls and after aminotriazole administered alone.

Treatment	No. of animals	Total microsomal phospholipid[a]	Total microsomal protein[a]
0.9% NaCl	5	45 ± 12[b]	92 ± 6[b]
Phenobarbital	5	81 ± 27[c]	148 ± 10[c]
Phenobarbital + aminotriazole	8	76 ± 21[c]	132 ± 16[c]
Aminotriazole	4	47 ± 5[b]	99 ± 8[b]

[a] Values are expressed as milligrams per gram of liver × [(liver weight/body weight) × 100].

[b] Differences between means in the same column, designated by the same superscript, are not statistically significant. Other differences are significant ($p < 0.05$).

[c] Differences between means in the same column, designated by the same superscript, are not statistically significant. Other differences are significant ($p < 0.05$).

outer membranes, but with invaginations of the matrix (Fig. 5).

Although the increase in smooth endoplasmic reticulum following treatment with phenobarbital and aminotriazole appeared somewhat less prominent (Fig. 4) than with phenobarbital alone (Fig. 1), microsomal phospholipid was increased similarly in both groups (Table 4). When compared with NaCl controls, aminotriazole alone did not alter the phospholipid levels or the amount of endoplasmic reticulum.

DISCUSSION

In the present study, aminotriazole, an inhibitor of heme biosynthesis (2), was administered *in vivo*, concomitantly with phenobarbital, in order to determine whether interference with heme biosynthesis would decrease the formation of induced membranes of smooth endoplasmic reticulum or alter the structure of membranes formed.

The effect of aminotriazole on the inhibition of heme biosynthesis is not an "all-or-none" phenomenon. Aminotriazole administered alone produces at most a 50% decrease in cytochrome P-450 levels 16 hr after administration (2, 4). After 4 days of aminotriazole treatment alone, the reduction in cytochrome P-450 is no more than 30%

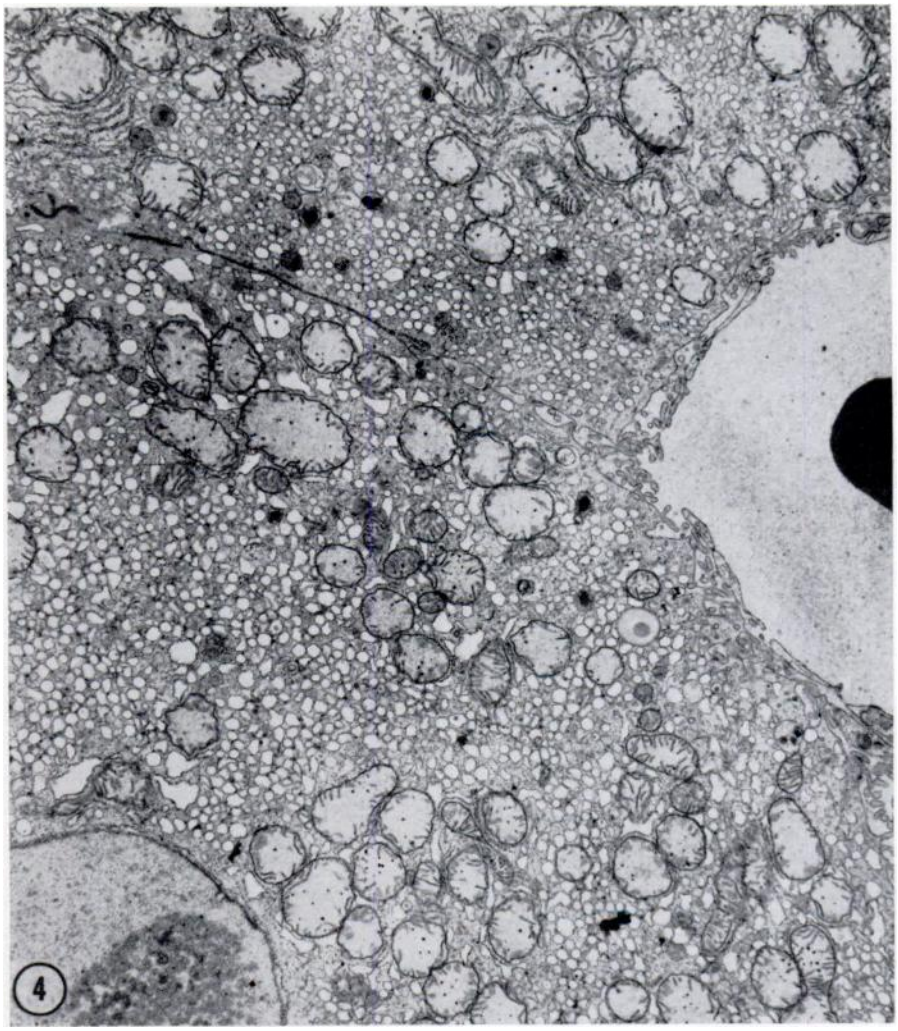

FIG. 4. *Hepatocyte from rat treated with phenobarbital and aminotriazole*
Increased smooth endoplasmic reticulum and depletion of glycogen are apparent. × 10,000.

(Table 2). The mechanism by which this inhibitory effect is counteracted is not known.

Aminotriazole administered together with phenobarbital for 4 days partially counteracted the increases in drug-hydroxylating activity and in cytochrome P-450 obtained with phenobarbital alone. Combined treatment with phenobarbital and aminotriazole, when compared to phenobarbital alone, effects a similar increase in the amount of smooth membranes of the endoplasmic reticulum, but produces a much smaller increase in cytochrome P-450 and drug-hydroxylating activity.

Aminotriazole does not affect microsomal protein synthesis, as measured by the induction of the specific non-heme flavoprotein cytochrome *c* reductase (2) or the nonspecific incorporation of leucine into microsomal protein (16). In the present study, the quantity of microsomal protein after combined phenobarbital and aminotriazole treatment was similar to that found after phenobarbital treatment alone, although the absolute values were always somewhat lower in the former case. Recently available methods for the isolation of cytochrome P-450 (17) may make it possible to determine whether this small but constant decrease in microsomal

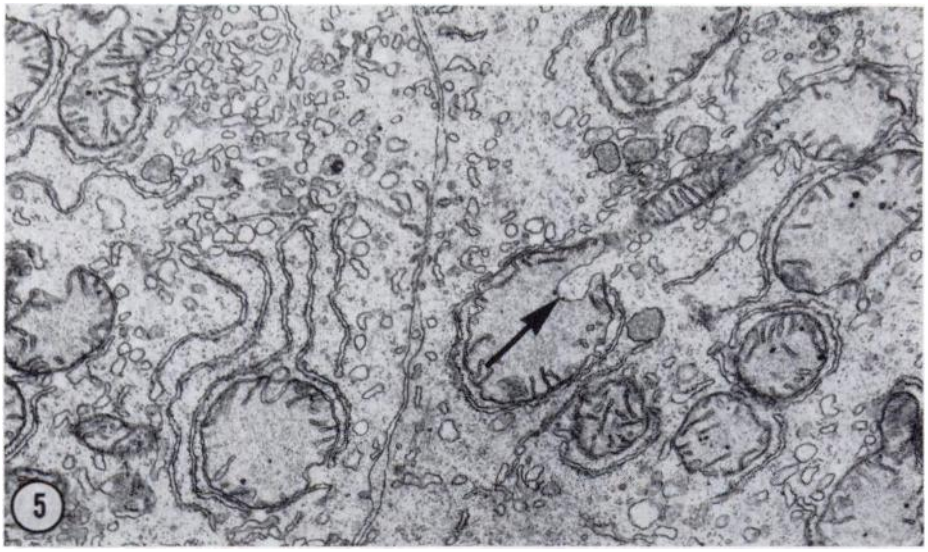

FIG. 5. *Mitochondria in hepatocytes from rat treated with phenobarbital and aminotriazole*
Focal loss of outer membrane and invagination of the matrix (arrow) have occurred. × 30,000.

protein reflects the absence of the "protein" moiety of the hemoprotein P-450.

The association of cytochrome P-450 with the membranes of hepatic endoplasmic reticulum is well recognized, but the exact way in which the cytochrome is related to the membranes is not known. The increase in the lipid constituents of induced microsomal membranes may be due to synthesis of new membranes, decreased catabolism, or a combination of both mechanisms (18, 19). The difficulty which many workers have experienced in solubilizing cytochrome P-450 and the necessity of intact lipid for type I drug metabolism (20), have suggested that the cytochrome is firmly attached to the membrane and possibly is an integral part of its structure.

The present study indicates that in the presence of an inducer such as phenobarbital, interference with cytochrome P-450 biosynthesis by aminotriazole does not appreciably alter induction of the formation of membranes of hepatic endoplasmic reticulum as observed by electron microscopy and measured by microsomal phospholipid. This suggests that the levels of cytochrome P-450 and the lipoprotein portion of the endoplasmic reticulum membrane may be controlled separately.

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