Puromycin Inhibition of Enzyme Induction by 3-Methylcholanthrene and Phenobarbital

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Administration of phenobarbital or 3-methylcholanthrene to animals markedly enhances the activity of several reduced triphosphopyridine nucleotide-dependent enzyme systems in liver microsomes (1-5). Although the resistance of these enzyme systems to solubilization and purification has prevented definitive studies on the mechanism by which the enhanced activity occurs, such as has been done with immunological studies on the induced synthesis of tryptophan pyrrolase (6) and tyrosine-\(\alpha\)-ketoglutarate transaminase (7), the available evidence strongly suggests that the increased enzyme activity is the result of induced synthesis of more enzyme protein (1-4, 8). To study this problem further, we have utilized the antibiotic puromycin, which blocks the transfer of soluble RNA-bound amino acid to microsomal protein both in vivo and in vitro (9-11). The results of the studies presented here demonstrate that puromycin completely blocks the ability of 3-methylcholanthrene or phenobarbital to increase the activity of an enzyme system in liver microsomes that oxidatively \(N\)-demethylates 3-methyl-4-monomethylaminoazobenzene.

EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats weighing 45 to 50 g were fed a synthetic diet containing 22% casein, 0.5% L-cystine, 5% corn oil, 4.5% salt mixture, 68% glucose, and high levels of all the known vitamins. The rats were treated with intraperitoneal injections of sodium phenobarbital in 0.23 ml of 0.9% sodium chloride solution, with 3-methylcholanthrene in 0.25 ml of corn oil or with puromycin dihydrochloride (Nutritional Biochemical Corporation) dissolved in 0.1 ml of the buffered NaCl solution described by Gorski et al. (9). Control animals were given injections of the appropriate vehicles. The rats were killed by decapitation, and 10% liver homogenates were prepared in 0.25 M sucrose solution at 2\(^\circ\). Homogenates were assayed for ability to \(N\)-demethylate 3-methyl-4-monomethylaminoazobenzene\(^\#\) to 3-methyl-4-aminoazobenzene in the presence of excess cofactors as previously described (12).

RESULTS

Puromycin Inhibition of Enzyme Induction by 3-Methylcholanthrene—Hourly intraperitoneal injections of 20 or 30 mg per kg of puromycin dihydrochloride into rats completely blocked 3-methylcholanthrene-induced increases in aminoazo dye \(N\)-demethylase activity, and injections of 10 mg per kg caused a 61% block (Fig. 1). When puromycin was given 10 hours after the administration of 3-methylcholanthrene and at a time when enzyme activity was increasing rapidly, the antibiotic completely prevented further increases in activity, and \(N\)-demethylase activity was maintained at a partially elevated level (Fig. 2). The administration of puromycin for 10 hours to control rats or to rats treated 10 hours previously with 3-methylcholanthrene decreased enzyme activity by only 14 and 6%, respectively, suggesting that the aminoazo dye \(N\)-demethylase is slowly degraded in vivo with a half-life greater than 24 hours.

The addition in vitro of 6.1 \(\times\) \(10^{-4}\) M or 1.2 \(\times\) \(10^{-4}\) M puromycin dihydrochloride to incubation mixtures containing liver homogenate from 3-methylcholanthrene-treated rats resulted in a 41 and 17% inhibition of aminoazo dye \(N\)-demethylase activity, respectively. However, the inhibitory action of puromycin in vitro appears unrelated to the action of the antibiotic in preventing increases in enzyme activity in vivo since the hourly injections of control or 3-methylcholanthrene-treated rats with 20 mg per kg of puromycin dihydrochloride for 10 hours had little or no inhibitory effect on \(N\)-demethylase activity. Furthermore, the administration of a single 120 mg per kg dose of puromycin dihydrochloride to 3-methylcholanthrene-treated rats 30 minutes prior to death had no effect on demethylase activity.

Puromycin Inhibition of Enzyme Induction by Phenobarbital—Phenobarbital differs from 3-methylcholanthrene as an inducer of microsomal enzyme activity in that phenobarbital slowly and nonselectively stimulates the activity of many microsomal TPNH-dependent enzymes over a 3 to 4 day period, whereas 3-methylcholanthrene and other polycyclic hydrocarbons rapidly and selectively stimulate the activity of only a few microsomal TPNH-dependent enzymes. Since phenobarbital administration stimulates the activity of the aminoazo dye \(N\)-demethylase system slowly and with a lag period of a few hours, hourly puromycin injections were initiated 7 hours after the administration of phenobarbital and were continued for 12 hours. The hourly injection of 20 mg per kg of puromycin dihydrochloride completely blocked increases in enzyme activity whereas injections of 10 mg per kg resulted in a 48% block (Fig. 3).

Stimulatory Effect of Phenobarbital and Other Drugs on Synthesis of Protein in Liver Microsomes—The administration of several drugs that increase the activity of TPNH-dependent enzymes in liver microsomes (3) also alters hepatic protein metabolism so that the ratio of synthesis to degradation is increased (Table I). This effect of drugs results in a net increase in microsomal protein. Phenobarbital was the most potent agent in exerting this effect. The intraperitoneal injection of 37 mg per kg of sodium

\(^\#\) We are grateful to Dr. James A. Miller (University of Wisconsin) for gifts of 3-methyl-4-monomethylaminoazobenzene and 3-methyl-4-aminoazobenzene.
followed 5 minutes later by hourly injection of 2 mg of 3-methylcholanthrene (MC) or vehicle was
injected. Each value represents the average and range obtained with the livers of 3 rats. The
intraperitoneal injection of 20 mg per kg of puromycin dihydrochloride (PU) dissolved in buff-
ered 0.9% NaCl solution (BS). The control rats received hourly injections of the buffered NaCl solution.
Enzyme activity is expressed as micrograms of 3-methyl-4-aminoazobenzene (3-methyl-
AB) formed in 12 minutes by homogenate from 50 mg of liver. All injections were given intraperitoneally. Each value represents the average and range obtained with the livers of 5 rats.

Group I was killed at zero time; Group II received a 2-mg injection of 3-methylcholanthrene (MC) at zero time and was killed at 10 hours; Group III received an injection of corn oil (CO) at zero time and was killed at 10 hours; Group IV received an injection of 3-methylcholanthrene at zero time, an injection of corn oil at 10 hours and hourly injections of buffered 0.9% NaCl solution (BS) from 10 to 20 hours; Group V received an injection of 3-methylcholanthrene at zero time, an injection of corn oil at 10 hours, and hourly injections of 20 mg per kg of puromycin dihydrochloride (PU) from 10 to 20 hours; Group VI received an injection of 3-methylcholanthrene at zero time, an injection of corn oil at 10 hours, and hourly injections of 10 mg per kg of puromycin dihydrochloride from 10 to 20 hours; Group VII received an injection of 3-methylcholanthrene at zero time, an injection of corn oil at 10 hours, and hourly injections of 20 mg per kg of puromycin dihydrochloride from 7 to 19 hours; Group VIII was given injections of 80 mg per kg of sodium phenobarbital at zero time, 40 mg per kg of sodium phenobarbital at 7 hours and hourly injections of buffered 0.9% NaCl solution (BS) from 7 to 19 hours; Group IX was treated by injection with NaCl solution from 7 to 19 hours; Group X was given injections of 80 mg per kg of sodium phenobarbital at zero time, 40 mg per kg of sodium phenobarbital at 7 hours and hourly injections of 20 mg per kg of sodium phenobarbital at 7 hours and hourly injections of 10 mg per kg of puromycin dihydrochloride (PU) from 7 to 19 hours; Group XI was given injections of 80 mg per kg of sodium phenobarbital at zero time, 40 mg per kg of sodium phenobarbital at 7 hours and hourly injections of 20 mg per kg of sodium phenobarbital at 7 hours and hourly injections of 10 mg per kg of puromycin dihydrochloride from 7 to 19 hours; Group XII was treated with 0.9% NaCl solution at zero time, the same at 7 hours, and with hourly injections of 20 mg per kg of puromycin dihydrochloride from 7 to 19 hours; Group XIII was given injections of 0.9% NaCl solution at zero time and at 7 hours, and hourly injections of 10 mg per kg of puromycin dihydrochloride from 7 to 19 hours; Group XIV was treated by injection with NaCl solution at zero time and at 7 hours, and with hourly injections of 20 mg per kg of puromycin dihydrochloride from 7 to 19 hours. Enzyme activity is expressed as the micrograms of 3-methyl-4-aminoazobenzene formed in 12 minutes by homogenate from 50 mg of liver. All injections were given intraperitoneally. Each value represents the average and standard error obtained with the livers of 5 rats.

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In addition to an increase in liver size, microsomal protein per g of liver was increased 39%, resulting in a 70 to 80% increase in microsomal protein per rat. Histological studies showed that the increased size of the liver was due to increased numbers of cells, produced by a stimulatory effect of phenobarbital on liver mitosis and cell division. The intraperitoneal injection of 20 mg per kg of 3-methylcholanthrene did not increase the amount of microsomal protein in the liver at 24 hours. However, the administration of 10 to 20 mg per kg of this hydrocarbon each day for 4 days did stimulate liver growth, resulting in a 20 to 40% increase in microsomal or total liver protein. Microsomal protein per g of liver, however, was not consistently increased by treatment of rats with 3-methylcholanthrene.
Lowry et al. (22) with bovine plasma albumin as a protein salt administered. Protein analyses were done by the method of as the free drug. Dosages were calculated on the basis of the salt administered. Protein analyses were done by the method of Lowry et al. (22) with bovine plasma albumin as a protein standard. Each value represents the average and standard error of 4 to 8 experiments. Pooled livers from 5 animals were used for each experiment.

### Table I

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Daily dose</th>
<th>Microsomal protein</th>
<th>Increase</th>
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<tbody>
<tr>
<td>Control</td>
<td>mg/kg</td>
<td>mg/g wet weight liver</td>
<td>%</td>
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<tr>
<td>Phenobarbital</td>
<td>75</td>
<td>24.3 ± 0.7</td>
<td>39</td>
</tr>
<tr>
<td>Chlorkyclizine</td>
<td>24</td>
<td>23.1 ± 0.2</td>
<td>32</td>
</tr>
<tr>
<td>Orphenadrine</td>
<td>50</td>
<td>22.3 ± 0.5</td>
<td>27</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>110</td>
<td>21.3 ± 0.2</td>
<td>22</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The ability of puromycin to block the increases in the activity of aminooazo dye N-demethylase that are caused by 3-methylcholanthrene or phenobarbital suggests that these agents increase demethylase activity by stimulating the formation of more enzyme molecules from amino acids. These results are consistent with earlier studies that also indicate the induction of more enzyme protein (1–4, 8). The effects of 3-methylcholanthrene and phenobarbital are not mediated through hormones of the adrenal or pituitary gland, for 3-methylcholanthrene has been shown to induce the synthesis of the aminooazo dye N-demethylase system in hypophysectomized or adrenalectomized rats, and phenobarbital has been shown to induce the synthesis of barbiturate-metabolizing enzymes in either hypophysectomized or adrenalectomized-castrated rats (1, 13).

Phenobarbital, chlorkyclizine, orphenadrine, and phenylbutazone not only induce the synthesis of aminooazo dye N-demethylase (3) but also exert a more general anabolic action on the synthesis of many TPNH-dependent enzymes in liver microsomes (3, 13–15). Studies with the electron microscope (16) have recently shown that phenobarbital administration stimulates the formation of smooth membranes of the hepatic endoplasmic reticulum, which is where the TPNH-dependent oxidative enzymes are localized (17), and it is likely that the large increases in microsomal protein that we have observed are due to an increased production of smooth membranes of the endoplasmic reticulum. In contrast to phenobarbital, 3-methylcholanthrene and other polycyclic hydrocarbons selectively induce the synthesis of only a few microsomal enzymes (3, 14, 18, 19) without increasing the amount of measurable microsomal protein per g of liver. Although 3-methylcholanthrene does not increase microsomal protein per g of liver, the polycyclic hydrocarbons, like phenobarbital, have a potent stimulatory effect on liver growth and the synthesis of total liver protein, and earlier studies have suggested a relationship between the ability of polycyclic hydrocarbons to stimulate liver growth and to induce microsomal enzyme formation (4).

Studies by Gelboin and Sokoloff (8) with 3-methylcholanthrene and phenobarbital and by Von der Decken and Hultin (20) with 3-methylcholanthrene have shown that the marked effect of these agents on the synthesis of microsomal enzymes in vivo was paralleled by increased incorporation in vitro of amino acids into microsomal protein. More detailed studies by Gelboin and Sokoloff showed that 3-methylcholanthrene administration stimulated the incorporation of soluble RNA-bound amino acids into liver protein suggesting that this hydrocarbon was not acting on the reactions leading to activated amino acid but was acting on steps between transfer RNA and the formation of protein on the ribosome (8). Gelboin and Blackburn (21) have recently presented evidence that 3-methylcholanthrene stimulates the incorporation of amino acid into ribosomal protein by both increasing the formation of messenger RNA and by increasing the number of amino acid incorporating sites in the microsomes. These investigators (21) have shown that actinomycin D, an agent that selectively blocks the DNA directed synthesis of RNA, also blocks increases in the activity of 3,4-benzpyrene hydroxylase caused by 3-methylcholanthrene administration, and unpublished observations in our laboratory have shown that actinomycin D blocks the increases in the activity of aminooazo dye N-demethylase that are caused by 3-methylcholanthrene or phenobarbital administration. These results raise the possibility that 3-methylcholanthrene and phenobarbital may accelerate the DNA directed synthesis of RNA molecules that function as template for the synthesis of aminooazo dye N-demethylase enzyme on the ribosome. Additional studies on the effects of 3-methylcholanthrene and phenobarbital on the ribosomal incorporation sites and on messenger RNA formation should throw further light on the mechanisms by which these agents induce the synthesis of microsomal enzymes and stimulate the formation of large amounts of microsomal protein.

**SUMMARY**

The administration of puromycin to rats completely blocked increases in the activity of aminooazo dye N-demethylase caused by 3-methylcholanthrene or phenobarbital, providing evidence for the synthesis of more enzyme protein. Phenobarbital and certain other inducers of the aminooazo dye N-demethylase system were potent stimulators of net synthesis of liver microsomal protein.

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**REFERENCES**

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