The Stimulatory Effect of 3-Methylcholanthrene on Benzpyrene Hydroxylase Activity in Several Rat Tissues: Inhibition by Actinomycin D and Puromycin*

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SUMMARY

The presence of benzpyrene hydroxylase activity in the small intestine, lung, and kidney of the normal rat has been shown by quantitative assay and compared with the level of activity in the liver. The intraperitoneal injection of methylcholanthrene increased the level of activity found in each of the four tissues examined. The stimulation by methylcholanthrene was inhibited by the simultaneous administration of puromycin or actinomycin D, suggesting that the changes in enzyme level are dependent on protein and RNA synthesis in these tissues. Possible implications to a theory of carcinogenesis are discussed.

A single intraperitoneal injection of methylcholanthrene (MC) or 3,4-benzpyrene increases the activity of several rat liver microsomal enzyme systems (4–9, 11, 12, 14). The activity of one of these, benzpyrene hydroxylase, was shown by Conney et al. (7) to be stimulated to a marked degree by MC pretreatment. Recently, Wattenberg and Leong (21) have demonstrated histochemically the presence of benzpyrene hydroxylase in rat kidney, adrenal, lung, testis, and thyroid and have measured quantitatively its presence in small intestine (22). These authors also found that MC treatment increased the benzpyrene hydroxylase activity of each of the tissues in which the enzyme was found in the normal rat with the exception of the adrenal.

Studies in our laboratory on the mechanism by which MC stimulates certain enzyme activities in the rat liver have shown that MC increases the rate of microsomal amino acid incorporation when either free amino acids or soluble RNA-bound amino acids are used as precursors (14). The MC stimulation of microsomal amino acid incorporation is paralleled by an MC-induced increase in the number of active microsomal amino acid incorporation sites (13) and an increase in the amount and specific stimulatory activity of nuclear RNA (19). These findings are consistent with the hypothesis that the MC-induced changes in enzyme activity are due to enzyme synthesis mediated through the production of messenger RNA. In this report data are presented on the benzpyrene hydroxylase activity of several rat tissues, the stimulatory effect of MC on these levels, and the inhibition of the MC effect by puromycin, an inhibitor of protein synthesis, and by actinomycin D, an inhibitor of DNA-dependent RNA synthesis (16).

MATERIALS AND METHODS

All rats were males of the Sprague-Dawley strain and were maintained on Purina Laboratory Chow prior to the experiment. During the experiment the rats were fasted. Groups of four to seven rats weighing between 40 and 50 gm. were given injections intraperitoneally of 1 mg. of methylcholanthrene in 0.25 ml. corn oil or corn oil only. After the indicated time periods, the rats were killed by decapitation, the tissues excised, and a 10 per cent homogenate made in 0.25 M sucrose solution with a Potter-Elvehjem type all-glass homogenizer. In Experiments 1 and 2 (Charts 2–5) the tissues from three rats were homogenized and assayed individually. In Experiments 3–5 (Charts 2–5) and in the experiment of Chart 1 the tissues from groups of four to seven rats were homogenized, pooled, and assayed. In the experiment in Chart 1 and in Experiments 3–5 (Chart 4) random sections of small intestine were homogenized. In Experiment 2 (Chart 4) sections of small intestine 4–10 cm. from the stomach were utilized.

The benzpyrene hydroxylase assay was performed by a modification of the method of Wattenberg et al. (21). The reaction mixture contained 50 µmoles sodium phosphate buffer, pH 7.4, 12 µmoles glucose 6-phosphate, 1.26 µmoles NADPH, 0.75 µmoles NADH, 120 µmoles nicotinamide, 2.0 µmoles ATP, 5.0 µmoles MgCl₂, 50 µmoles KCl, and enzyme equivalent to 5–30 mg. wet tissue in 0.25 M sucrose solution. Final volume was 3.0 ml. Fifty µg. of 3,4-benzpyrene in 100 µl. of methanol was added, and the mixture was incubated for 12 minutes at
37°C. The reaction was stopped with 3.0 ml. of cold acetone. Nine ml. of petroleum ether (B.P.-30°-60°C.) was added, and the flasks were incubated for 10 minutes at 37°C. The mixture was shaken and then stored in the dark at 4°C. for 24-48 hours. An aliquot of the organic phase (0.5-3.0 ml.) was extracted with 10.0 ml. of 1 N NaOH. The fluorescence was determined within 5 minutes after extraction in an Aminco-Bowman spectrofluorometer with the activation wave length set at 400 µm and the fluorescence read at 522 µm. The fluorescence was standardized with a sample of 8-hydroxy benzpyrene. The extracted metabolites represent a mixture of hydroxylated products. The spectrum of the mixture indicates that 8-OH benzpyrene is the major metabolite, although significant amounts of other hydroxylated metabolites are present. At least two levels of enzyme were assayed in duplicate. The amount of extractable material in the incubation medium with no tissue added was equivalent to 0.7-1.3 µg. of 8-OH benzpyrene. The addition of tissue at zero time increased this value by no more than the equivalent of 0.1 µg. of 8-OH benzpyrene. Zero time control values containing tissue and incubation medium were subtracted from the values for the incubated flasks. A unit of enzyme is the amount of enzyme producing a fluorescence change at 522 µm equivalent to the production of 1 µg. of 8-OH benzpyrene per minute.

RESULTS

Benzpyrene hydroxylase activity in tissues of normal and MC-treated rats.—Chart 1 shows the amount of benzpyrene hydroxylase present in liver, kidney, lung, and small intestine of normal 40- to 50-gm. male rats. The amount of enzyme present per wet weight of tissue is of the order of 1-10 per cent of that present in liver.

The intraperitoneal injection of MC increased the level of benzpyrene hydroxylase activity in each of the tissues examined. In the experiment shown in Chart 1, at 7 and 14 hours after the administration of 1.0 mg. of methylcholanthrene the benzpyrene hydroxylase activity was increased in liver approximately four- and ninefold, in lung 1¾- and fourfold, in kidney six- and nineteen-fold, and in intestine thirteen- and 30-fold.

In other experiments (Charts 2-5) in which activity was assayed 6-10 hours after MC was administered the amount of stimulation induced by MC in each tissue varied over a wide range. Each tissue tested always exhibited increased activity after MC treatment, but the amount of stimulation varied as much as three- to fourfold in a given tissue in different experiments over a similar time period. The most variable response was observed with lung tissue. The reason for this variability is not known.

The effect of actinomycin D on the MC-induced increases in benzpyrene hydroxylase.—Charts 2-5 show the increase in benzpyrene hydroxylase activity in liver, kidney, small intestine, and lung after the administration of MC and the inhibition of the stimulation by the simultaneous administration of actinomycin D. In liver (Chart 2) actinomycin D inhibited the MC-induced increases in benzpyrene...
hydroxylase activity in each of four experiments. In Experiment 2 the inhibition was complete. In the other experiments the MC response was inhibited from 40 to 80 per cent by actinomycin D. In kidney (Chart 3) actinomycin inhibited over 95 per cent of the MC stimulation in three experiments (Exps. 1, 2, 4) and 50 per cent in the fourth experiment (Exp. 3). In three experiments (Chart 4) the MC-induced increase in benzpyrene hydroxylase in small intestine was inhibited by 100, 80, and 58 per cent by the administration of actinomycin. In lung actinomycin was least effective as an inhibitor of the MC-stimulated increase in enzyme activity. In three experiments with lung (Chart 5) actinomycin inhibition was 44, 65, and 80 per cent. Thus, in each tissue at least part of

![Chart 3](image)

**Chart 3.**—The effect of actinomycin D and puromycin on the MC stimulation of benzpyrene hydroxylase activity in rat kidney. Conditions for each experiment are the same as in Chart 2 and "Methods."

![Chart 4](image)

**Chart 4.**—The effect of actinomycin D and puromycin on the MC stimulation of benzpyrene hydroxylase activity in rat small intestine. Conditions for each experiment are the same as in Chart 2 and "Methods."

the MC induction of benzpyrene hydroxylase was found to be dependent on an actinomycin-sensitive reaction. The lack of complete prevention of the stimulatory effect of MC in certain experiments may be due to incompete-ness of the inhibition of cellular RNA synthesis by actinomycin D. In some tissues this may be due to an impermeability to the inhibitor. Another possibility is the formation of some active enzyme through a mechanism independent of RNA synthesis. The same amount of actinomycin D as used in Experiment 2 (Charts 2-5) did not appreciably affect the basal level of enzyme in any of the tissues tested in the untreated rats or high levels of the liver enzyme in rats treated with MC.

**The effect of puromycin on the methylcholanthrene stimulation of benzpyrene hydroxylase activity.**—Charts 2-5 show the effect of the simultaneous administration of puromycin on the MC-induced changes in benzpyrene hydroxylase activity. In each of the four tissues tested simultaneous in vivo administration of puromycin inhibits the MC stimulation.

The relative amount of inhibition varied among the tissues tested. Thus, in liver (Chart 2) and intestine (Chart 4) the inhibitory effect of puromycin was greater than 90 per cent in every experiment. In kidney (Chart 3), the inhibition ranged from 37 to 90 per cent in four experiments, and in lung the inhibition ranged from 31 to 50 per cent in four experiments. The differences in the degree of inhibition may represent variability in the absorption of the inhibitor or the effectiveness of the inhibition in different tissues. Puromycin given alone over the same time period as in Experiment 2 did not alter the basal level of enzyme in any tissue or affect a high level of liver enzyme activity previously induced by methylcholanthrene. These results indicate that at least part of the stimulatory effect of methylcholanthrene in each of the tissues tested was prevented by puromycin, an inhibitor of protein synthesis.
DISCUSSION

The results show the relative levels of benzpyrene hydroxylase activity in normal rat liver, kidney, small intestine, and lung. The liver contained considerably more activity per wet weight of tissue than did the other organs tested and probably represents the major site of this type of hydroxylation in the rat. However, the other tissues exhibiting activity may, under certain conditions, represent significant physiological sites for the hydroxylation of aromatic compounds. Thus, the lungs may be an important site of hydroxylation of polycyclic compounds entering the organism through the respiratory tract, and the small intestine may play a role in the hydroxylation of polycyclic compounds ingested in the diet.

Conney et al. (4–7) and others (8, 9) have surveyed the effect of the in vivo administration of MC and benzpyrene on a variety of liver microsomal enzymes. These investigations demonstrated that treatment with polycyclic hydrocarbons increases the activity of certain microsomal enzymes, whereas other microsomal enzyme activities are either depressed or remain unchanged. Wattenberg and Leong (21) have shown by histochemical methods that MC induces benzpyrene hydroxylase activity in several rat tissues. Our results confirm these findings by quantitative assay. Gilman and Conney (15) have recently demonstrated the stimulatory effect of MC on amino azo dye demethylase in a number of rat tissues in addition to liver. Dutton and Stevenson (10) showed increased glucuronide synthesis in the skin of mice treated with 3,4-benzpyrene. Thus, the increase in enzyme activity induced by polycyclic hydrocarbons is not unique to the liver but may represent a regulatory mechanism common to many mammalian tissues.

Investigations on the mechanism of the MC-induced liver microsomal changes suggest that the increase in certain enzyme activities is due to enzyme synthesis which is mediated through a production of messenger-RNA. The evidence supporting this hypothesis is as follows: (a) MC increases microsomal amino acid incorporation (14)—this is reflected in an increased number of active microsomal amino acid incorporation sites (13); (b) MC increases the amount of RNA in rat liver nuclei and enhances its capacity to stimulate amino acid incorporation (19); (c) the increase in liver amino azo dye demethylase induced by MC is prevented by the simultaneous administration of puromycin; and (d) the results presented here show that increases in benzpyrene hydroxylase induced by MC in several tissues are inhibited by both puromycin and actinomycin D.

Boyland (3) has discussed a possible relationship between enzyme induction and carcinogenesis. Several of the compounds that induce microsomal enzymes are potent carcinogens, whereas others are noncarcinogenic (1, 4). In one study Conney has shown that the enzyme changes induced by methylcholanthrene, a potent carcinogen, are different from the pattern of changes induced by phenobarbital, a noncarcinogenic drug (4). In other studies Arcos et al. (1) found no positive correlation between the carcinogenicity of the hydrocarbon tested and its capacity to induce amino azo dye demethylase. The relationship, if any, between enzyme induction and carcinogenesis must await a detailed analysis of the specific patterns of enzyme changes induced by various carcinogens versus those changes induced by noncarcinogens.

Single large doses of MC produce tumors in the mammary gland of rats (17), and MC is an initiator of the carcinogenic process in the two-stage mechanism of skin carcinogenesis of Berenblum (2). These findings suggest that the early biochemical events subsequent to MC administration may be intimately related to the conversion of a normal cell to either the malignant or premalignant condition. Some of the early biochemical events after MC treatment have been discussed here. The MC-induced changes in microsomal enzyme activities appear to be due to an alteration in the protein synthetic apparatus and in the expression of specific genetic information. In view of the recent discoveries on the mechanism of control of enzyme synthesis by gene repression and derepression (18) and the apparent effect of MC on the enzyme-synthesizing system of rat liver, it is possible to speculate on a relationship between MC-induced changes in enzyme synthesis and chemical carcinogenesis. It is possible that one of the early events in carcinogenesis is an alteration of genetic expression—i.e., the carcinogen may either repress or de-repress specific gene information, thereby resulting in either enzyme repression or induction. If the induced changes alter the synthesis of enzymes controlling growth or cell division, the result may be the production of a malignant cell or a malignant cell precursor. Another possibility, as suggested by Monod and Jacob (20), is that the alteration of genetic expression in a given tissue may change the internal environment of the tissue so as to make more probable the selection and survival of a mutant tumor cell.

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REFERENCES


A. H. Conney, personal communication.


Erratum

The following correction should be made in the article by Elion et al., "Relationship between Metabolic Fates and Antitumor Activities of Thiopurines," Cancer Research, 23:1207-17, 1963:

In the legend of Chart 1, page 1213, the line broken by squares represents 6-chloropurine alone; the line broken by circles represents 6-chloropurine + HPP (20 mg/kg, I.P.).
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