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Induction of Drug Metabolism

IV. Relative Abilities of Polycyclic Hydrocarbons to Increase Levels of Microsomal 3-Methyl-4-methylaminoazobenzene *N*-Demethylase Activity and Cytochrome P_1-450

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SUMMARY

Previous studies from this laboratory showed that the administration of 3-methylcholanthrene and 3,4-benzpyrene caused the formation of cytochrome P_1-450, which differs from cytochrome P-450 in its substrate specificity, in the difference spectra it produces with ethyl isocyanide, and in its drug-binding properties. Six polycyclic hydrocarbons, previously shown to range in inductive capacities from no activity to very high activity, were administered to rats. The various degrees of induction of 3-methyl-4-methylaminoazobenzene *N*-demethylase that resulted were related directly to changes in ethyl isocyanide difference spectra and aniline binding difference spectra of microsomal hemoprotein, two measurements which reflect the presence of cytochrome P_1-450. When a maximally stimulatory dose of a polycyclic hydrocarbon was given, it caused finite increases in microsomal 3-methyl-4-methylaminoazobenzene *N*-demethylase activity and cytochrome P_1-450 content, whether it was a poor, intermediate, or potent inducing agent.

INTRODUCTION

Previous studies from this laboratory (1–5) led to the proposal that the administration of 3-methylcholanthrene or 3,4-benzpyrene to rats caused the formation of a new form of microsomal hemoprotein, cytochrome P_1-450, which appears to differ from cytochrome P-450 in certain of its biochemical and physical properties. Cytochrome P-450 functions in the *N*-demethylation of ethylmorphine; cytochrome P_1-450 either does not do so or functions much less efficiently. Both cytochromes participate in the *N*-demethylation of 3-methyl-4-methylaminoazobenzene. The difference spectrum observed when ethyl isocyanide combines with cytochrome P-450 differs from that seen with cytochrome P_1-450 in the relative magnitude of the Soret peaks at 430 and 455 mμ (1, 3). The relative sizes of the two peaks depend on the pH of the medium in which the microsomes are suspended (6). At low pH the 430 mμ peak predominates; at high pH the 455 mμ peak predominates. At pH 7.4, the 430 and 455 mμ peaks are of equal size when hepatic microsomes from untreated and phenobarbital-treated rats are employed, but when microsomes from 3-methylcholanthrene-treated rats are used, the peaks are of equal size at pH 6.9 (1). Remmer and co-workers (7) and Imai and Sato (8) showed that drugs combine with microsomal hemoprotein in two different ways.

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In general, they can be grouped into two classes: type I compounds, which give a difference spectrum with a trough at 419–425 mμ and an absorption peak at 385–390 mμ, and type II compounds, which give a spectrum with an absorption peak at 426–435 mμ and a trough at 390–405 mμ. Unlike cytochrome P-450, which possesses both binding sites, cytochrome P_1-450 seems to be devoid of the type I binding site, or largely so (4, 5). Finally, the absorption maximum of cytochrome P_1-450 is at a slightly lower wavelength than that of cytochrome P-450 (9, 10), and the absolute spectra of the two cytochromes differ (10).

Arcos, Conney, and Buu-Hoi (11) studied more than 50 polycyclic aromatic hydrocarbons for their ability to induce the *N*-demethylation of 3-MMAB.[2] Depending upon their molecular size and degree of planarity, the various polycyclic hydrocarbons showed various degrees of inductive capacity, ranging from no activity to very high activity. If polycyclic hydrocarbons cause the formation of cytochrome P_1-450, as judged from the change in effect of pH on the absorption maxima of the hemoprotein when it is combined with ethyl isocyanide, then the degrees of this change should relate directly to the potencies of the hydrocarbons as inducers of 3-MMAB *N*-demethylase. The studies of Arcos and co-workers permitted the selection of compounds to test this possibility. Because the polycyclic hydrocarbons cause an increase in type II binding without an increase in type I binding, it seemed likely that the degrees of stimulation of the *N*-demethylation of 3-MMAB caused by various polycyclic hydrocarbons of different inductive potencies would also relate directly to increases in the type II binding site. The current studies were performed to determine the relationship between the stimulation of the microsomal *N*-demethylation of 3-MMAB and the formation of cytochrome P_1-450 as determined by changes in the spectral and drug-binding properties of microsomal hemoprotein.

[2] The abbreviations used are: 3-MMAB, 3-methyl-4-methylaminoazobenzene; 3-MC, 3-methylcholanthrene; 9,10-DMBA, 9,10-dimethyl-1,2-benzanthracene.

MATERIALS AND METHODS

The following polycyclic hydrocarbons were employed: 3-methylcholanthrene, 1,2-benzpyrene, 3,4-benzpyrene, 1,2-benzanthracene, 9,10-dimethyl-1,2-benzanthracene, and 1,2,3,4-dibenzanthracene. They were recrystallized three times from benzene and ethanol, and their purity was tested using gas-liquid chromatography.

Male Holtzman rats (80–90 g) were maintained on a 27% protein (casein) diet (Nutritional Biochemicals Corporation) for 4–7 days prior to each experiment. The polycyclic hydrocarbons (18.6, 37.2, or 186 μmoles/kg) were administered intraperitoneally in 0.5 ml of corn oil. Twenty-four hours later the livers were removed, and microsomes (9000 × *g* supernatant fluid or 105,000 × *g* pellet) were prepared as described previously (1, 2). These fractions were used on the same day they were prepared.

The 3-MMAB *N*-demethylase activity of the 9000 × *g* supernatant fraction was determined by measuring the rate of formaldehyde formation, as described previously (1, 2). The difference spectra that result when aniline binds to microsomal hemoprotein (105,000 × *g* pellet) were determined by the method of Remmer and coworkers (7), using a Shimadzu model MPS 50 spectrophotometer. The difference spectra seen when microsomal hemoprotein combines with ethyl isocyanide were determined at several pH values, as described previously (1, 3). The two curves that result when the heights of the 430 mμ and 455 mμ peaks are plotted against pH cross at the pH where the two peaks are the same height. This intercept is referred to as the "pH intercept." Protein content of the liver preparations was determined by the method of Lowry *et al.* (12).

RESULTS

In Fig. 1 it can be seen that 1,2,3,4-dibenzanthracene, 3-methylcholanthrene, and 3,4-benzpyrene are potent inducers of 3-methyl-4-methylaminoazobenzene *N*-demethylation; 1,2-benzanthracene is an intermediate inducer; and 9,10-dimethyl-1,2-benzanthracene is a poor inducer. 1,2-

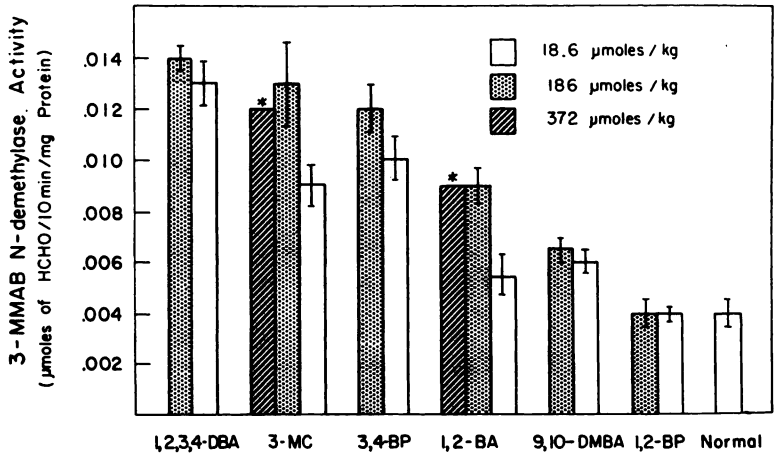

FIG. 1. *Effect of several polycyclic hydrocarbons on 3-MMAB N-demethylation*

1,2,3,4-Dibenzanthracene (1,2,3,4-DBA), 3-methylcholanthrene (3-MC), 3,4-benzpyrene (3,4-BP), 1,2-benzanthracene (1,2-BA), 9,10-dimethyl-1,2-benzanthracene (9,10-DMBA), and 1,2-benzpyrene (1,2-BP) were administered intraperitoneally in 0.5 ml of corn oil 24 hr prior to the determination of the 3-MMAB *N*-demethylase activity of hepatic microsomes. 3-MMAB at a concentration of 2×10^{-4} M was incubated for 10 min with 9000 × *g* supernatant fraction equivalent to 10–25% of fresh liver. Activity is expressed in micromoles of formaldehyde formed in 10 min per milligram of protein contained in the 9000 × *g* supernatant fraction. In the experiments indicated by asterisks, two animals were used; five or six animals were used in all other experiments.

Benzpyrene is a symmetrical molecule; it failed to show inductive activity. These results agree with those of Arcos and coworkers (11). In most cases the inductive effect obtained with 18.6 μmoles of polycyclic hydrocarbon per kilogram was about the same as that seen with 186 μmoles/kg. In those cases when differences were observed, as with 3-methylcholanthrene and 1,2-benzanthracene, a dose of 372 μmoles/kg was employed, but this did not result in a greater inductive effect than that obtained with the 186 μmoles/kg dose. It is concluded that maximum effects were obtained with all of the compounds at the 186 μmoles/kg dose.

That the maximal degree of induction obtainable with the various polycyclic hydrocarbons was not dose-related suggested the possibility that a compound with low inductive capacity might in some way interfere with the effectiveness of a potent inducer. If, for example, each of the compounds was capable of fully occupying the site which initiates induction, it might be expected that the less active compounds would prevent to some degree the occupancy of that site by the more active compounds, which thus would be prevented from exerting their full effects. That this is not the case is seen in Table 1. The simultaneous administration of various doses of 9,10-DMBA, a poor inducing agent, did not prevent 3-MC, a potent inducer, from eliciting its full effect, nor did the administration of 9,10-DMBA for 3 days prior to the administration of 3-MC alter the effectiveness of 3-MC.

Microsomal preparations from rats receiving various doses of different polycyclic hydrocarbons were assayed for their 3-MMAB *N*-demethylase activities and for their cytochrome P_1-450 contents as reflected by the pH intercept of the 430 and 455 mμ peaks formed with ethyl isocyanide. The excellent correlation between decreasing pH intercepts and increasing *N*-demethylase activities is shown in Fig. 2. The study was repeated, except that the change in the aniline binding spectrum was employed as a measure of microsomal cytochrome P_1-450 content rather than the pH intercept. The very good correlation between increases in

TABLE 1

Inductive effects on 3-MMAB N-demethylation produced by 3-MC and 9,10-DMBA administered singly and in combination

Treatment[a]		3-MMAB *N*-demethylation[b]
3-MC	9,10-DMBA	
μmoles/kg		*μmole HCHO/mg/10 min ± SE*
0	0	0.0040 ± 0.00034 (7)[c]
0	186	0.0065 ± 0.00054 (5)
46.5	0	0.0160 ± 0.00025 (3)
46.5	186	0.0164 ± 0.0013 (3)
93	0	0.0199 ± 0.00117 (3)
93	186	0.0196 ± 0.00164 (3)
186	0	0.0184 ± 0.00036 (3)
186	186	0.0200 ± 0.00045 (3)
186	0[d]	0.0139 ± 0.00025 (5)
0	186[e]	0.0087 ± 0.0018 (3)
186	186[f]	0.0136 (2)

[a] Daily for 4 days unless otherwise indicated.

[b] The conditions for measuring 3-MMAB *N*-demethylation are given in the legend to Fig. 1.

[c] Number of animals is given in parentheses.

[d] 3-MC injected for 2 days.

[e] 9,10-DMBA injected for 5 days.

[f] 9,10-DMBA injected alone for 3 days, and then 9,10-DMBA and 3-MC injected simultaneously for 2 more days.

aniline binding and increases in 3-MMAB *N*-demethylase activity is shown in Fig. 3.

When the pH intercept and aniline binding values are normalized to a 0–100 scale, where 0 is the control value and 100 the maximum change in pH intercept or the maximum height of the aniline difference spectrum, and these values are plotted against 3-MMAB *N*-demethylase activity, two linear plots satisfying almost identical equations are obtained (Fig. 4), thus clearly establishing the relationship between the spectral and binding properties of cytochrome P_1-450 and 3-MMAB *N*-demethylase activity.

DISCUSSION

These studies further demonstrate that polycyclic hydrocarbons induce a form of cytochrome P-450 (cytochrome P_1-450) which differs from that normally preponderant in hepatic microsomes. By employing the binding of aniline and ethyl isocyanide to microsomal hemoprotein as indirect measures of the amount of cytochrome P_1-450 in microsomes, a direct correlation was shown between the increases in 3-MMAB *N*-demethylase activity caused by the administration of polycyclic hydrocarbons of different inductive capacities and the amount of cytochrome P_1-450 that was formed in response to the treatment. The investigation does not explain how the polycyclic hydrocarbons produce their inductive effects, or why one is more active than another. Of the several ways in which these inducing agents might act, the possibility must be considered that the com-

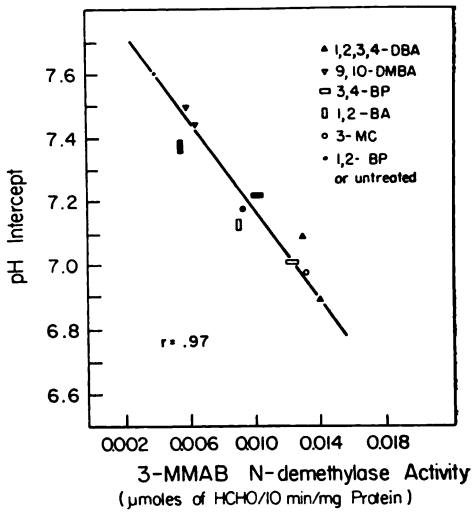

FIG. 2. *Effect of several polycyclic hydrocarbons on pH intercept of ethyl isocyanide difference spectra of microsomal hemoprotein*

Microsomes (resuspended 105,000 × *g* pellet) and 9000 × *g* supernatant fraction were obtained from livers removed from rats 24 hr after a single intraperitoneal injection of 18.6 μmoles (solid symbols) or 186 μmoles (open symbols) of compound per kilogram. The pH intercept is defined as the pH at which the 430 and 455 mμ difference spectrum maxima are the same height. The suspensions (resuspended 105,000 × *g* pellet) used in the spectral studies contained 1.3–2.6 mg of protein per milliliter. The 9000 × *g* supernatant fraction was used in the *N*-demethylation studies as described for Fig. 1. Each point represents the mean of five animals. The line was drawn from a computed least-squares linear regression analysis of the data. *r* is the correlation coefficient calculated from the same data. For abbreviations, see the legend to Fig. 1.

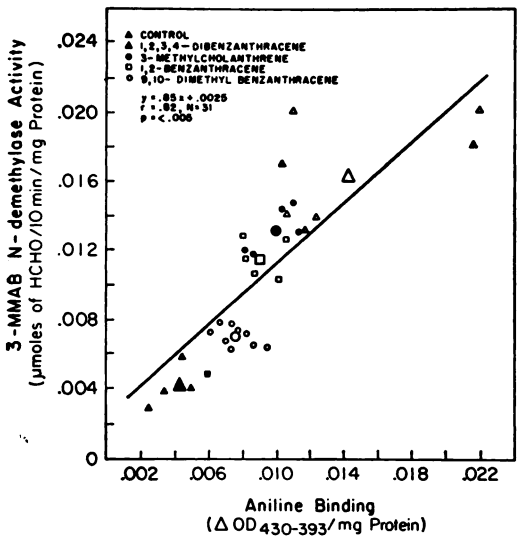

FIG. 3. *Relationship among 3-MMAB metabolism, aniline binding, and inductive capacity of several polycyclic hydrocarbons*

Microsomes (resuspended 105,000 × *g* pellet) and 9000 × *g* supernatant fraction were obtained from livers removed from rats 24 hr after a single intraperitoneal injection of hydrocarbon (186 μmoles/kg). The suspensions (resuspended 105,000 × *g* pellet) used in the binding studies contained 1.0–1.6 mg of protein per milliliter. Concentrations of aniline used were 3.2 or 6.3 mM, the higher concentration being used when the inductive effect was great; maximum binding spectra were always obtained with both concentrations of aniline. The 9000 × *g* supernatant fraction was used in the *N*-demethylation studies as described for Fig. 1. The large symbols are averages of corresponding small symbols, which represent values obtained from individual animals. The line was drawn and *r* was calculated as described for Fig. 2.

pounds or their metabolites may combine with microsomal cytochrome P-450, either directly or during the synthesis of the hemoprotein. If the agent itself is incorporated into the hemoprotein, the differences in the activities of the compounds might be explained by their relative reactivities with the hemoprotein, either indirectly or after initiation of an inductive process involving hemoprotein synthesis. If the metabolites of these compounds are the effective agents rather than the compounds per se, the same explanation would be relevant, but the degree to which each of the agents was metabolized would introduce an additional consideration; the rate of biotransformation of the agents might explain the differences in activities. Whatever the explanation, it is of some interest that each of the agents appeared capable of producing only a given degree of induction of microsomal 3-MMAB *N*-demethylase activity or cytochrome P_1-450 content, regardless of the size of the dose. In most of the current studies results were observed 24 hr after a single injection of the agent, but even after 4 days of administration, 9,10-DMBA, a poor inducing agent, did not produce as great an inductive effect as 3-MC, a potent inducer (Table 1).

Cytochrome P_1-450 is more limited than cytochrome P-450 in its reactivity with drug substrates, probably because it is essentially lacking in the type I binding site, and thus might be considered an aberrant cytochrome. That polycyclic hydrocarbons should produce such an aberration is not incompatible with certain concepts that would attempt to explain the carcinogenicity of many of these compounds. However, Arcos and co-workers (11) have drawn attention to the fact that many polycyclic hydro-

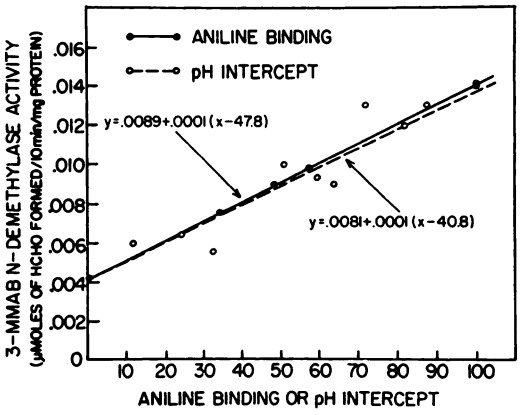

FIG. 4. *Correlation between microsomal aniline binding, pH intercept of ethyl isocyanide difference spectra, and 3-MMAB N-demethylase activity*

The data from Figs. 2 and 3 have been normalized to a 0–100 scale, where 0 is the control value and 100 the maximum change in pH intercept or the maximum height of the aniline difference spectrum.

carbons which are potent carcinogens are poor inducing agents, and vice versa.

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