EFFECT OF AFLATOXIN AND BENZPYRENE PRETREATMENT UPON THE KINETICS OF BENZPYRENE HYDROXYLASE

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It is well recognized that many foreign compounds can cause increased liver microsomal enzyme activity when administered to animals. Several workers have reported that this increased activity results from a net synthesis of microsomal enzyme protein based upon the observation that such "induction" is blocked by the administration of a protein inhibitor such as ethionine (Conney et al., 1957; Conney et al., 1960; Fujimoto et al., 1961). Not all compounds appear to be alike in their abilities to increase microsomal enzyme activities. Phenobarbital, for example, has been shown to stimulate the enzymatic conversion of many drugs whereas aromatic polycyclic hydrocarbons affect a more limited number of drugs (Conney, 1967). In order to clarify the mechanism leading to enzyme "induction" elicited by the administration of polycyclic aromatic hydrocarbons, experiments were undertaken to study the effects of the pretreatment of rats with 3,4-benzpyrene (BP) and aflatoxin (B_1) upon the subsequent kinetic behavior of the microsomal BP hydroxylase as a model system.

Aflatoxin B_1, the well-known carcinogen produced by Aspergillus flavus, was chosen as one of the polycyclic hydrocarbons for comparison, both because of its ability to inhibit protein synthesis (Smith, 1963) as well as its known metabolism to a hydroxylated derivative (deIongh et al., 1964; Holzapfel et al., 1966). Because of the influence of age on the inducibility of microsomal enzymes (Murphy et al., 1958), rats of two different age groups were used. It is shown in the present study that the "induction" of BP hydroxylase results primarily from a qualitative change

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in the existing enzyme rather than an increase in enzyme synthesis. Furthermore, younger animals show this response to a greater degree.

METHODS AND MATERIALS

Tritium-labelled 3,4-benzpyrene (specific activity = 4 mC/μmole) was obtained from Nuclear-Chicago; NADP, glucose-6-P (disodium salt) and yeast glucose-6-P dehydrogenase were obtained from Calbiochem. Aflatoxin B_1 was isolated and purified according to the method of Hanna and Campbell (1967).

Experiments were conducted using Sprague-Dawley derived rats of two age groups. Experiment I was composed of three groups, each of which was comprised of three 52-day old rats; whereas in Experiment II, three groups of rats, each composed of nine 35-day old rats, were used. In both experiments one group was injected (I.P.) with B_1 (67 μg/100 gm body weight), a second group was injected (I.P.) with BP (1mg/100 gm body weight) and a third group was injected with a corresponding volume of the diluent of 20% Tween 80 in saline (0.3 ml/100 gm body weight). Upon arrival, all rats were fed commercial lab chow ad libitum for 4 days prior to treatment and were sacrificed 24 hours following treatment. Livers were excised, weighed, chilled, pooled for each group, and homogenized in 2-3 volumes of pH 7.4 phosphate buffer. Homogenates were centrifuged at 20,000 x g for 15 minutes to remove nuclei and mitochondria. The microsomal fractions were collected as pellets of the 105,000 x g centrifugation according to the method of Silverman and Talalay (1967). The pellets were then suspended in the phosphate buffer to give a concentration of microsomes equivalent to 1 gm of liver/ml. Protein was estimated by the method of Lowry et al. (1951).

Tritium-labelled BP was diluted with cold BP to a specific activity of 120 cpm/m mole. Appropriate dilutions were made from this solution so that 0.05 ml of ^{3}H-BP in ethanol was added to each reaction mixture. Therefore, each 1.5 ml of reaction mixture contained 0.64-0.70 mg of microsomal protein, 3 mM glucose-6-P, .48 mM NADP, 2 units of glucose-6-P dehydrogenase as well as the ^{3}H-BP. Incubations were carried out for the appropriate times in a Dubnoff metabolic shaker at

37°C. The reaction was stopped by adding 3.5 ml of 0.25 N KOH in 50% ethanol. Unmetabolized BP was extracted with hexane and counted according to the method of Silverman and Talalay (1967).

Velocities were found to be linear during a 10-minute incubation period. Initial velocities were recorded for different substrate concentrations and the data were analyzed using the computer program of Cleland, $V = V_m A/K+A$ (Cleland, 1963). Each concentration was run in quadruplicate and the initial velocity was determined.

RESULTS AND DISCUSSION

The effect of B_1 and BP pretreatments upon the microsomal protein concentration are shown in Table 1. Kinetic parameters for both experiments are shown in Table 2. It should be emphasized that throughout this discussion of the kinetic studies the Michaelis constants and maximum velocities are assumed to be "apparent" values. The Lineweaver-Burke plot for both sets of data are shown in Figures 1 and 2.

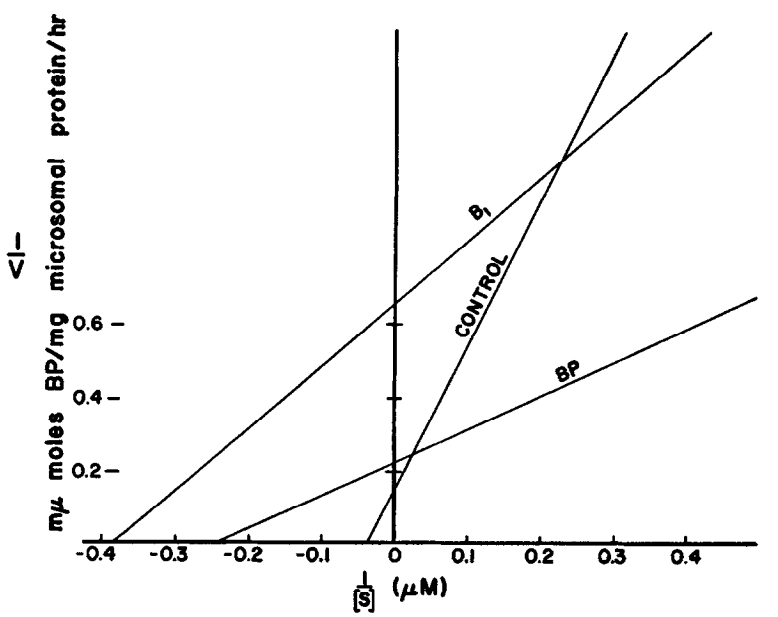

Figure 1. Effect of pretreatment on benzpyrene hydroxylase activity of liver microsomes of 35-day old rats. Please see footnotes c and d of Table 2 for the number of animals representing each plot. Each plot represents the BP-hydroxylase activity measured in the microsomes of animals pretreated 24 hours prior to killing.

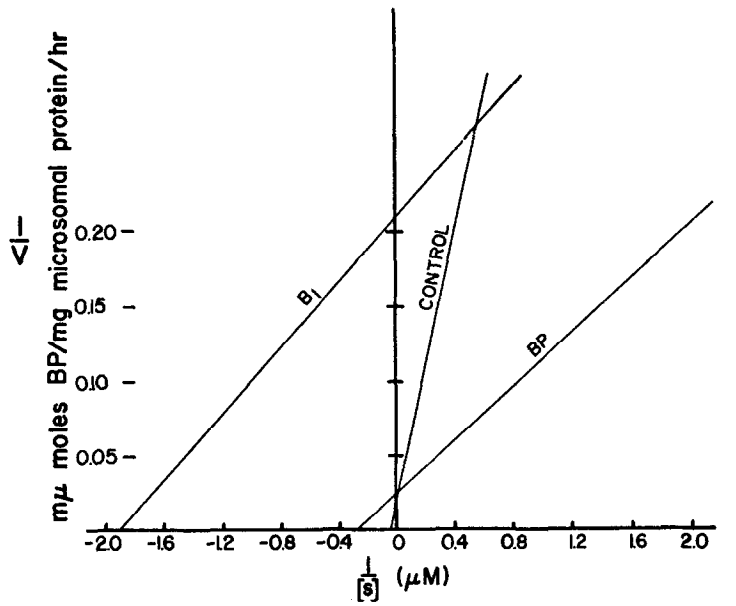

Figure 2. Effect of pretreatment on benzpyrene hydroxylase activity of liver microsomes of 52-day old rats. Please see footnotes c and d of Table 2 for the number of animals representing each plot. Each plot represents the BP-hydroxylase activity measured in the microsomes of animals pretreated 24 hours prior to killing.

TABLE 1. Effect of Pretreatment with Aflatoxin B_1 and Benzpyrene on Liver Microsomal Protein

Group (No.)	mg/gm[a]	Mean Weight of Liver (gm)
Control (9)	33.2	5.72
Aflatoxin B_1 (8)	30.8	5.57
Benzpyrene (9)	35.2	6.00

[a] Concentration of microsomal protein in mg/gm of pooled liver.

TABLE 2. EFFECT OF BENZPYRENE AND AFLATOXIN B_1 PRETREATMENT UPON THE BENZPYRENE HYDROXYLASE ACTIVITY OF RAT LIVER MICROSOMES [g]

Parameter	Control	B_1 Pretreatment	BP Pretreatment
35-day old rats:[c]			
V_{max}[a]	55.6 ± 48.6[e]	4.86 ± 0.62[f]	47.8 ± 2.4
K_m[b]	60.6 ± 52.4[e]	0.53 ± 0.56[f]	3.21 ± 0.48
$K/_V$	1.09 ± 0.09[e]	0.110 ± 0.104[f]	0.067 ± 0.007
52-day old rats:[d]			
V_{max}[a]	67.0 ± 12.2	14.9 ± 1.6	42.6 ± 3.4
K_m[b]	28.6 ± 10.0	2.61 ± 1.48	4.04 ± 1.43
$K/_V$	0.428 ± 0.073	0.175 ± 0.085	0.095 ± 0.027

[a] Disappearance of BP in mμmoles/mg microsomal protein/hr.

[b] Substrate concentration in μM.

[c] Each value represents 3 and 5 substrate concentrations (each run in quadruplicate) for pretreated groups and control group, respectively.

[d] Each value represents 5 substrate concentrations, each run in quadruplicate.

[e] The relatively large S.E. for V_{max} and K_m is due to the choice of substrate concentrations being too low rather than highly variable data (Cleland, 1967) as indicated by the S.E. of the slope.

[f] The high S.E. for the K_m and $K/_V$ terms are due to the choice of substrate concentrations being too high rather than highly variable data (Cleland, 1967).

[g] Although the variability of some of the data leaves something to be desired, the very marked experimental responses, which are consistent in both studies, enable the conclusions in the text to be made.

Pretreatment with B_1 was found to slightly decrease microsomal protein. Such an observation is consistent with earlier observations concerning its protein inhibitory effects. Since the effect of B_1 on the V_{max} of BP hydroxylase (Table 2 and Figures 1 and 2) is a marked reduction, any interpretation of this change in terms of a decrease in protein concentration would have to further assume that a fairly specific inhibition of protein synthesis occurred. The data resulting from pretreatment with BP would also require the assumption of specificity in the protein changes since the total microsomal protein increased slightly even though the V_{max} decreased slightly.

As an alternative, one could propose that the reduction in V_{max} with BP treatment results from a decrease in the rate constant(s) for the catalytic step(s). Although the kinetic mechanism for this enzyme is not yet known, some general suggestions can be put forth on the basis of general enzyme kinetic theory. A decrease in the rate constants involved in the breakdown of the enzyme-substrate complex(es) would usually lead to a lower V_{max}. If this occurred, it would probably result in lowering the K_m for the substrate also, thereby augmenting any decrease in the K_m produced by a decrease in the dissociation constant which might occur simultaneously.

If, according to many of the previous reports, the induction of microsomal enzymes caused by prior treatment of animals with foreign compounds is solely due to increased protein synthesis, the Michaelis constant should theoretically remain constant and the V_{max} should increase. Many of the workers who have concluded that such induction is caused by a net enzyme protein synthesis have based their conclusions on the reversal of the inductive effect with a protein inhibitor such as ethionine. However, if less than saturating substrate concentrations were used, the ethionine reversal effect would not have detected alterations in rate constants and therefore could be misleading. Nevertheless, Rubin et al. (1964) found that phenobarbital administration increased the V_{max} of ethylmorphine N-demethylase, chlorpromazine sulfoxidase and hexobarbital oxidase but did not influence the K_m or the susceptibility of these enzyme systems to various inhibitors. Netter et al. (1964) reported that both phenobarbital and 3,4-benzpyrene increased

the V_{max} of p-nitroanisole O-demethylase without influencing the K_m. Although both of these reports could be explained on the premise that inductions of a number of microsomal enzyme systems are in fact the result of increased enzyme protein formation, they do not exclude the possibility of modifications of enzyme systems involved in the metabolism of the inducer compound used in the pretreatment.

In the results reported in this study, however, both BP and B_1 pretreatment significantly lowers the K_m in each case whereas in no case is V_{max} increased. This demonstrates that pretreatment with either one of these compounds results in some sort of activation of the hydroxylase enzyme system rather than a net increase in enzyme protein. Similar observations were recently reported by Alverez et al. (1968) with respect to the effect of pretreatment with 3-methylcholanthrene upon the K_m value of BP hydroxylase.

It should also be noted that the pretreatment with either B_1 or BP caused a 10-fold and 3-fold greater reduction, respectively, in Michaelis constants for the younger animals. This observation agrees with the comments by Conney (1967) but would appear to represent the greater ability of younger animals to develop a qualitative rather than quantitative modification of the enzyme system.

REFERENCES

Alvares, A. P., Schilling, G. R. and Kuntzman, R., Biochem. Biophys. Res. Commun. 30: 588 (1968).

Cleland, W. W., Nature 198: 463 (1963).

Cleland, W. W., Adv. Enzymol. 29: 1 (1967).

Conney, A. H., Miller, E. C., and Miller, J. A., J. Biol. Chem. 228: 753 (1957).

Conney, A. H., Davison, C., Gastel, R., and Burns, J. J., J. Pharmacol. Exptl. Therap. 130: 1 (1960).

Conney, A. H., Pharmacol. Revs. 19: 317 (1967).

de Iongh, H., Vles, R. O., and van Pelt, J. G., Nature 202: 466 (1964).

Fujimoto, J. M. and Plaa, G. L., J. Pharmacol. Exptl. Therap. 131: 282 (1961).

Hanna, L. K. and Campbell, T. C., Proc. Va. J. Sci. 18: 171 (1967).

Holzapfel, C. W., Steyn, P. S. and Purchase, I. F. H.,Tetrahedron Let., No. 25: 2799 (1966).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. T., J. Biol. Chem. 193: 265 (1951).

Netter, K. J. and Seidel, G., J. Pharmacol. Exptl. Therap. 146: 61 (1964).

Rubin, A., Tephly, T. R., and Mannering, G. J., Biochem. Pharmacol. 13: 1007 (1964).

Silverman, D. A. and Talalay, P., Molec. Pharmacol 3: 90 (1967).

Smith, R. H., Biochem. J. 88: 50P (1963).