Biochemical Pharmacology, Vol. 19, pp. 1729–1735. Pergamon Press. 1970. Printed in Great Britain

A KINETIC APPROACH TO A STUDY OF THE INDUCTION OF RAT LIVER MICROSOMAL HYDROXYLASE AFTER PRETREATMENT WITH 3,4-BENZPYRENE AND AFLATOXIN B_1*

H. L. GURTOO† and T.C. CAMPBELL‡

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, College of Agriculture, Blacksburg, Va., U. S. A.

(*Received* 5 *July* 1969; *accepted* 3 *October* 1969)

Abstract—The administration of 3,4-benzpyrene (BP) increases rat liver microsomal hydroxylase activity either by promoting the synthesis of additional enzyme protein or by activation of existing enzyme as measured by alterations in the apparent maximal velocity (V_{max}) or apparent Michaelis Constant (K_m) respectively. The values of the kinetic constants of the induced enzyme are dependent on the amount of inducer given, and by comparing the dosage-dependent trends with the parameters for the uninduced control enzyme, it is possible to demonstrate interaction between the inducer and the enzyme. Ethionine given at the same time as benzpyrene prevents an increase in the V_{max} but permits a reduction of the K_m to 10 per cent of the original control level, allowing a calculation of the extent of BP binding to the existing enzyme. In the absence of ethionine, after BP administration, more enzyme is synthesized, thereby decreasing the proportion of BP-bound enzyme. When a second dose of BP is given 24 hr after an initial dose, the K_m is reduced from the 24-hr enzyme preparation, whereas the V_{max} is maintained at an equivalent level, which indicates additional interaction between the inducer and the enzyme synthesized from 0 to 24 hr. This suggests that the binding of BP is to a site other than the active site of the enzyme. Aflatoxin B_1 (AB_1) also possesses similar abilities to stimulate hydroxylase synthesis and activation (decrease in K_m), but the effects are considerably compromised by its inhibitory effects on protein synthesis.

THE EFFECT of chemical carcinogens and other xenobiotics upon hepatic microsomal enzymes which metabolize these foreign compounds is becoming increasingly important, since the activities of these "drug metabolizing" enzymes effect the resulting physiological properties of the parent compounds. Several factors such as age, diet, sex, species, strain and diurnal period influence microsomal activities,[1–3] although the most significant effects are usually demonstrated as a result of the administration of xenobiotic compounds.[1] Generally speaking, this increased activity of microsomal enzymes after the administration of a foreign compound has been thought to result from increased protein synthesis since it can be blocked by ethionine[4]

* Supported, in part, by USPHS Grant No. 1 RO1 ES00336-01A1 from the Division of Environmental Health Sciences and Grant No. 377 from the Nutrition Foundation, and submitted in partial fulfillment of the requirements for the Ph.D. degree by the senior author.

† Present address: Department of Pharmacology, Yale University School of Medicine, New Haven, Conn.

‡ Please submit requests for reprints to Dr. T. C. Campbell.

and puromycin,[5] as well as actinomycin[6] which blocks the DNA-dependent RNA synthetic reaction. However, recent communications[7, 8] have shown that synthesis *de novo* of microsomal enzyme(s) alone cannot explain the increased activity of microsomal benzpyrene hydroxylase after the administration of 3,4-benzpyrene (BP), aflatoxin B_1 (AB_1)* or 3-methylcholanthrene, as evidenced by decreased Michaelis constants (K_m). Both reports[7, 8] have shown that there are qualitative alterations of BP hydroxylase, as demonstrated by the decreased K_m values.

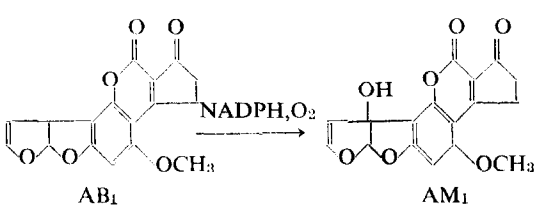
The present experiments were undertaken to examine the effects of the administration of the carcinogenic compounds, 3,4-benzpyrene (BP) and aflatoxin B_1 (AB_1), upon the kinetic behavior of rat liver microsomal hydroxylase with benzpyrene used as the substrate. By measuring the apparent values for the maximal velocity (V_{max}) and Michaelis constant (K_m) of this enzyme, we were able to determine whether induced enzyme activity results entirely from an increase in protein synthesis.

AB_1 is an extremely potent hepatocarcinogen[9] and is reported to be ring hydroxylated.[10, 11] Using thin-layer chromatography we have found that like BP, AB_1 is ring hydroxylated to an aflatoxin derivative, aflatoxin M_1, as shown in Fig. 1. The hydroxylation reaction was found to be catalyzed by hepatic microsomes and to require NADPH and molecular O_2; under N_2, no product was formed. Thus, like the polycyclic hydrocarbon BP, AB_1 is probably metabolized by the same cytochrome P450-requiring mixed-function oxidase system as other xenobiotics.[12–20] Ability of AB_1 to act as an inducer of microsomal enzyme activity should be related both to its activity as inducer *per se*, as well as to its reported activity as an inhibitor of protein synthesis.[21, 22]

MATERIALS AND METHODS

Sprague–Dawley derived male rats weighing 160–200 g (45 days of age at the time of injection) were used. Weanling animals were obtained, caged individually, and fed a semipurified 20% casein diet for at least 10 days in order to insure adequate basal levels of the enzyme for the groups injected with aflatoxin. The percentage composition of the semipurified diet was sucrose, 69·6; casein, 20·0; corn oil, 4·0; minerals, 4·0; vitamins, 4·0; and L-methionine, 0·2. The mineral and vitamin mixtures were the Jones–Foster Salt Mixture and the Vitamin Diet Fortification Mixture of Nutritional Biochemicals Corp. respectively.

Animals were randomly assigned to groups and injected intraperitoneally with the respective dosages of AB_1 (isolated and purified by the method of Hanna and Campbell[23]) or BP. The vehicle used was 20% Tween 80 in saline and an injection volume of 6·0 ml/kg was employed. Ethionine (ET) was given at a level of 700 mg/kg together



AB_1 AM_1

FIG. 1. Metabolic transformation of aflatoxin B_1 to aflatoxin M_1 requiring NADPH and O_2.

* The name aflatoxin is given to a group (4) of metabolites produced by *Aspergillus flavus*. Aflatoxin B_1 is the predominant species. The structure of aflatoxin B_1 and its polar metabolite aflatoxin M_1, is shown in Fig. 1.

with the respective dose of carcinogen. All animals were killed by decapitation either 24 or 48 after injection.

Livers were quickly excised, weighed, chilled, pooled for each group and homogenized in 2–3 vol. of cold 0·05 M potassium phosphate buffer containing 0·25 M sucrose (pH 7·4). Microsomes were isolated as pellets of the 105,000 *g* centrifugation, according to the method of Silverman and Talalay.[24] The microsomal pellets were then suspended in fresh buffer to give a concentration of microsomes equivalent to 1 g liver/ml. Protein was determined by the method of Lowry *et al.*[25]

The ^{3}H-labeled BP was diluted with cold BP in ethanol to a specific activity of 1000 cpm/mμmole. Appropriate dilutions were made from this solution so that different concentrations of ^{3}H-BP in a volume of 0·05 ml of ethanol were added to each reaction mixture. A total of 1·50 ml of reaction mixture contained 3 mM glucose 6-phosphate, 0·48 mM NADP, 16·7 mM potassium phosphate buffer (pH 7·4), 83·5 mM sucrose, 1 mg bovine serum albumin, 2 units of glucose 6-P dehydrogenase, ^{3}H-BP in 0·05 ml ethanol, and the appropriate levels of microsomal protein in buffer (used to initiate the reaction; the medium was preincubated in order to convert NADP to NADPH). The amount of microsomal protein varied and depended on the pretreatment of the different groups of rats. In all cases, less than 0·6 mg microsomal protein/ml was used in the assay medium.[19] In all cases, the time course of the reactions was determined at each substrate concentration in order to insure the measurement of initial velocities; assay times were from 2 to 10 min. Duplicate incubations were carried out for each substrate concentration in a Dubnoff metabolic shaker at 37° in air. Reactions were stopped with 3·5 ml of 0·25 N KOH in 50% ethanol. Duplicate aliquots from each flask were extracted with hexane and the unmetabolized BP was counted in a Packard Tri-Carb scintillation counter, according to the method of Silverman and Talalay.[24] Initial velocities were recorded for different substrate concentrations and the data were analyzed according to the computer program of Cleland[26] ($V = V_{max}\, A/k + A$).

RESULTS

Table 1 shows the effect of different dosage levels of AB_1 and BP ± ethionine (ET) on the kinetic parameters V_{max} and K_m. In this table is also shown the effect on microsomal protein corresponding of different dosages of the inducer(s).

As shown in Table 1, 24 hr after a single injection of 10 mg BP/kg body weight, there is a 66 per cent decrease in the K_m for BP while there is an elevation of the maximal velocity (V_{max}) of BP hydroxylase. Accompanying this 14-fold increase in V_{max} was a 20 per cent increase in microsomal protein. Injection of twice this amount of BP (2X) caused a much greater increase in V_{max} (23-fold) while lowering the K_m decrease to 40 per cent of the normal value. Injection of 30 mg BP/kg body weight (3X) brought the K_m to the control level while further elevating the V_{max}, i.e. to 34 times higher than control. Accompanying the increase in the V_{max} was a corresponding increase in microsomal protein. After a single dose of BP, simultaneous injection of ethionine (0·7 g per kg body weight) blocked the increase in microsomal protein as well as the increase in BP hydroxylase (V_{max}). However, the K_m of BP was reduced to 10 per cent of the control value.

Assuming the effect of BP on V_{max} was because of the induction of new enzyme, as suggested from the results of ethionine treatment, its effect on K_m could only be

because of some effect of BP on the already present enzyme. Consequently, calculations were made to determine the amount of BP bound to the enzyme (Table 2). In these calculations it was assumed that after ethionine no new enzyme was synthesized, and that simultaneously given BP caused a maximal binding to the enzyme. The calculations were based on the arbitrary designation of the difference between the control K_m (2·95 μM for BP) and BP-ET K_m (0·27 μM for BP) as 100 per cent binding.

TABLE 1. HYDROXYLASE KINETIC PARAMETERS FOR RATS GIVEN VARIOUS DOSAGE LEVELS OF AFLATOXIN AND BENZPYRENE*

Group	Dosage (mg/kg, i.p.) Aflatoxin	Benzpyrene	Ethionine	K_m + S. E.†	V_{max} + S. E.†	Microsomal‡ protein
Control§				2·95 ± 0·66	11·0 ± 1·1	14·0
B_1 (1X)	0·67 (2·14)‖			0·78 ± 0·24	6·1 ± 0·6	15·7
B_1 (2X)	1·34 (4·28)			2·07 ± 0·68	11·8 ± 2·6	17·8
B_1 (3X)	2·01 (7·42)			1·82 ± 0·66	14·8 ± 3·4	19·4
BP (1X)		10·0 (39·4)‖		1·01 ± 0·13	182·0 ± 6·0	16·8
BP (2X)		20·0 (78·7)		1·80 ± 0·34	255·0 ± 23·0	19·0
BP (3X)		30·0 (118·1)		3·25 ± 0·86	378·0 ± 60·0	20·0
BP (1X)-ET		10·0	700	0·27 ± 0·11	14·6 ± 1·2	13·9

* A range of 0·4 to 8·0 μMBP was used to determine kinetic constants in each experiment. BP was found to be soluble in the medium at levels up to 30 μM without added albumin, and higher concentrations in the presence of 1 mg albumin per 1·5 ml reaction medium.

† Standard error obtained from computer analysis of data. K_m = μMBP; V_{max} = mμmoles BP disappearance per mg per hr.

‡ Mg per g pooled liver.

§ Administered diluent alone. All test solutions given in equivalent volumes. 1X = single dose level in one injection. 2X = double dose level in one injection. 3X = triple dose level in one injection.

‖ Numbers in parentheses show dosage levels expressed as mμmoles per kg.

TABLE 2. CALCULATED PERCENTAGE OF MICROSOMAL HYDROXYLASE BOUND TO INDUCER*

Group	Bound (%)
BP (1X)	75·0
BP (2X)	48·6
BP (3X)	0
B_1 (1X)	82·7
B_1 (2X)	39·6
B_1 (3X)	47·9
BP (1X)-ET	100·0

* An assumption is made that the BP(1X)-ET group represents maximum (100%) binding, with the BP (3X) group representing minimum (0%) binding.

Differences were represented as per cent of maximal binding. From these calculations it can be seen (Table 2) that after a single injection of BP, approximately 75 per cent of the enzyme was found 24 hr later, and with increasingly higher injections of the carcinogen, proportionately less of the total enzyme was BP-bound. Roughly similar results were obtained with different doses of AB_1, but with this compound the results were not as well defined.

In order to further characterize the effect on the K_m as being because of the binding

of the inducer to the enzyme, the experiments shown in Table 3 were performed. The results indicate that the K_m value of the *newly induced* BP hydroxylase can also be lowered by further BP injection, as seen with the un-induced enzyme [BP (1X) — ET, Table 1]. Rats were treated with 30 mg/kg body weight of BP and were killed 24 hr after the injection. The amount of BP remaining bound at this time was negligible, as determined from the K_m value (Tables 2 and 3). When an additional 10 mg/kg body weight of BP was given 24 hr after the BP (3X) induction dose, and the animals killed

TABLE 3. HYDROXYLASE KINETIC PARAMETERS AFTER ADMINISTRATION OF SUCCESSIVE DOSES OF BENZPYRENE

Group	Protocol schedule*			K_m + S. E.†	V_{max} + S. E.†	Microsomal protein‡
	0 hr	24 hr	48 hr			
BP (3X)	30·0*	Sacrifice		2·69 ± 0·55	345 ± 34	18·4
BP (3X) + BP (1X)	30·0	10·0	Sacrifice	1·00 ± 0·37	318 ± 40	17·1
BP (3X) + ET	30·0	700 Ethionine	Sacrifice	3·45 ± 1·21	384 ± 77	15·4

* All dosages expressed as mg per kg body weight. K_m = μMBP; V_{max} = mμmoles per mg per hr.
† Standard error obtained from computer analysis of data.
‡ Mg per g pooled liver.

24 hr later, the K_m value [Table 3, BP (3X) + BP (1X)] suggested that about 75 per cent of the enzyme was BP-bound. When ethionine was given 24 hr after BP (3X) injection, the induction of the enzyme obtained between 0–24 hr was not prevented (V_{max}-unchanged), but further new enzyme synthesis was blocked. The K_m for BP [BP (3X) + ET], however, was elevated markedly above that for the group of animals in which BP was injected 24 hr before sacrifice [BP (3X) — Table 3]. This indicated that the effect on K_m was not because of the synthesis of a different protein, but possibly because of a loss of something bound to the enzyme during the 48 hr before sacrifice.

DISCUSSION

The data presented are in agreement with previous reports[7, 8] that pretreatment of animals with polycyclic hydrocarbons affects the K_m as well as V_{max} of the hydroxylase system. The effect of the two compounds studied (BP and AB_1) was found to be dosage dependent. The greatest reduction in K_m was obtained by the lowest level of the compound given; successively larger doses had less effect on the K_m. In the case of AB_1, it has been reported[27] that 80·02 per cent of the injected dose was excreted within 24 hr and, furthermore, that within 2 hr nearly all of the 24-hr excretion was secreted into the gut via the bile. Thus, the effect of polycyclic hydrocarbons could depend on two phenomena: (a) the presence of the inducer plus (b) an effect begun by the presence of inducer but continuing even after its disappearance. The former would suggest some sort of interaction with the hydroxylase system, and the latter would suggest the activation of some synthetic mechanism.

This conclusion clearly supports the observation by Schenkman *et al.*[28] and Imai and Sato[29] who showed spectral evidence for substrate interaction with the mixed-function oxidase. Recently Schenkman *et al.*[30] found spectral evidence for the interaction of BP with this system. In addition, they were able to show the presence of

BP in liver microsomes of animals pretreated with BP; after extraction with organic solvent the absorption spectrum of BP was observed. Binding of substrate has been shown to markedly accelerate electron transport through the enzyme system,[31, 32] thereby indicating a facilitation of the oxidation of the xenobiotic in question.

In addition to the polycyclic hydrocarbon being bound to the enzyme active site and serving as a substrate, it also serves as an inducer. The data presented in Table 1 indicate dose dependency for elevation of the V_{max} which would indicate a rapid clearance of the inducer. Furthermore, this newly synthesized enzyme would most likely be exposed to very little inducer (metabolism would have reduced the level), and, therefore, should possess properties, i.e. K_m, characteristic of the original enzyme. The data presented suggest that the binding of the inducer compound is to some part of the enzyme other than the active site (a possible allosteric site), thereby causing a decrease in K_m. The K_m of the enzyme system measured 24 hr after a single injection of BP (1X) represents, then, a composite value, including the value for the original enzyme affected by the inducer (low K_m) and value for the new enzyme which appears after most of the inducer is gone (not affected by inducer, high K_m). Thus, when a larger level of inducer is given, which would raise the level of enzyme much higher, proportionately more unbound enzyme is obtained than when a lower amount of inducer is given. That this phenomenon actually occurs is shown by the effect of the inducer(s) in the presence of an inhibitor of new enzyme synthesis. The very low K_m value obtained would indicate the magnitude of allosteric binding uncontaminated by newly synthesized enzyme. Using this value as an example of 100 per cent binding, it was shown that the percent bound 24 hr after injection decreased with increasing dose because of the greater amount of enzyme synthesized. If the inducer were made available to the newly synthesized enzyme [Table 3, BP (3X) + BP (1X)] and the animals were killed 24 hr later, the K_m value decreased, indicating that the per cent of bound enzyme was increased markedly (an increase from negligible binding to 75 per cent bound). The value of K_m chosen for 100 per cent binding is only an assumption, valid only to the extent of inhibition of new enzyme synthesis by ethionine; the percentages, therefore, represent only minimal values. Such residual binding has been demonstrated by Schenkman *et al.*[30] who estimated from the amount of polycyclic hydrocarbon extracted from liver microsomes of induced animals that about 2–4 per cent of the enzyme was still polycyclic hydrocarbon-bound 40 hr after the compound was given.

Furthermore, since the K_m value obtained for the newly induced enzyme [BP (3X), Table 1 and BP (3X) + ET, Table 3] was slightly higher than the control K_m value, the latter may represent the binding of some endogenous substrate; this conclusion has also been reached by Schenkman *et al.*[30] on the basis of spectral studies. This is not at all surprising in view of the continual exposure of animals to a wide variety and quantity of food additive residues and environmental contaminants, as well as steroid substrates of endogenous origin. How much the K_m could be made to increase could not be determined from these data.

The single, double and triple doses of AB_1, on the other hand, altered the V_{max} values from the control value by a factor of only 0·6, 1·1 and 1·3 respectively (Table 1). The much lower responses of AB_1 in these experiments are most probably because of, in part, the well-known aflatoxin property of inhibiting protein synthesis.[21, 22] In addition, the much attenuated effect on V_{max} after treatment with AB_1 may also be

because of differences in dosages given. When expressed on molar basis (Table 1), the amount of AB_1 given was about $\frac{1}{18}$ that of BP. Equal amounts of AB_1 could not be used in view of the cost and toxicity.

Acknowledgements—The authors would like to thank Dr. R. E. Webb and Dr. K. M. Plowman for their advice and timely discussions. Both are on the faculty of this Department. We also gratefully acknowledge the technical assistance provided for part of this work by H. P. Misra of this Department.

REFERENCES

1. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
2. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150**, 279 (1965).
3. F. M. Radzialowski and W. F. Bousquet, *J. Pharmac. exp. Ther.* **163**, 229 (1968).
4. A. H. Conney, E. C. Miller and J. A. Miller, *J. biol. Chem.* **228**, 753 (1957).
5. A. H. Conney and A. G. Gilman, *J. biol. Chem.* **238**, 3682 (1963).
6. H. V. Gelboin and N. R. Blackburn, *Cancer Res.* **24**, 356 (1964).
7. H. L. Gurtoo, T. C. Campbell, R. E. Webb and K. M. Plowman, *Biochem. biophys. Res. Commun.* **31**, 588 (1968).
8. A. P. Alvares, G. R. Schilling and R. Kuntzman, *Biochem. biophys. Res. Commun.* **30**, 588 (1968).
9. M. C. Lancaster, F. P. Jenkins and J. McL. Philip, *Nature, Lond.* **192**, 1095 (1961).
10. H. de Iongh, R. O. Vles and J. G. Van Pelt, *Nature, Lond.* **202**, 466 (1964).
11. C. W. Holzapfel, P. S. Steyn and I. F. H. Purchase, *Tetrahedron Lett.* **25**, 2799 (1966).
12. R. W. Estabrook, D. Y. Cooper and O. Rosenthal, *Biochem. Z.* **338**, 741 (1963).
13. S. Orrenius and L. Ernster, *Biochem. biophys. Res. Commun.* **16**, 60 (1964).
14. H. Remmer, R. W. Estabrook, J. Schenkman and H. Greim, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **259**, 98 (1968).
15. E. Degkwitz, D. Luft, U. Pfeiffer and H. Staudinger, *Hoppe-Seyler's Z. physiol. Chem.* **349**, 465 (1968).
16. J. A. Castro, F. E. Green, P. Gigon, H. Sasame and J. R. Gillette, *Fedn Proc.* **27**, 350 (1968).
17. H. Remmer, J. Schenkman, R. W. Estabrook, H. Sasame, J. Gillette, S. Narasimhulu, D. Y. Cooper and O. Rosenthal, *Molec. Pharmac.* **2**, 187 (1966).
18. J. B. Schenkman and R. Sato, *Molec. Pharmac.* **4**, 613 (1968).
19. J. B. Schenkman, J. A. Ball and R. B. Estabrook, *Biochem. Pharmac.* **16**, 1071 (1967).
20. L. Ernster and S. Orrenius, *Fedn Proc.* **24**, 1190 (1965).
21. R. C. Shank and G. N. Wogan, *Fedn Proc.* **23**, 200 (1964).
22. R. H. Smith, *Biochem. J.* **88**, 50P (1963).
23. K. L. Hanna and T. C. Campbell, *J. Ass. off. analyt. Chem.* **51**, 1197 (1968).
24. D. A. Silverman and P. Talalay, *Molec. Pharmac.* **3**, 90 (1967).
25. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951)
26. W. W. Cleland, *Nature, Lond.* **198**, 463 (1963).
27. G. N. Wogan, G. S. Edwards and R. C. Shank, *Cancer Res.* **27**, 1729 (1967).
28. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
29. Y. Imai and R. Sato, *Eur. J. Biochem.* **1**, 419 (1967).
30. J. B. Schenkman, H. Greim, M. Zange and H. Remmer, *Biochim. biophys. Acta* **171**, 23 (1969).
31. P. L. Gigon, T. E. Gram and J. R. Gillette, *Molec. Pharmac.* **5**, 109 (1969).
32. J. B. Schenkman, *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1624 (1968).