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ANALYSIS OF THE ARYL HYDROCARBON HYDROXYLASE ASSAY*

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Abstract—The assay method and the properties of aryl hydrocarbon hydroxylase were studied with rat liver microsomes. The assay could be carried out by two methods: (1) the microsomes (initially at 15–20°) were pre-incubated at 37° and then benzo[a]pyrene and NADPH were added to initiate the assay; and (2) benzo[a]pyrene was added to microsomes at 15–20°, pre-incubated at 37°, and then NADPH was added to initiate the reaction. The aryl hydrocarbon hydroxylase activity obtained by method 1 was usually 60–120 per cent higher than that obtained by method 2. Several lines of study suggested that the microsomes can exist in two states. Binding of benzo[a]pyrene at temperatures lower than 20° would limit the microsomes to a low activity state whereas binding at temperatures above 25° would enable the microsomes to exist in a high activity state. The molecular basis of these activity states remains to be investigated. The effects of acetone and methanol, both having been widely used as the solvents for benzo[a]pyrene, on aryl hydrocarbon hydroxylase have also been studied. The aryl hydrocarbon hydroxylase activities of control and phenobarbital-induced microsomes were higher with acetone as the solvent for benzo[a]pyrene as compared with methanol. The hydroxylase activity of 3-methylcholanthrene-induced microsomes, however, was slightly higher with methanol.

The microsomal mono-oxygenase system catalyzes the biotransformation of aryl hydrocarbons, and this activity is usually referred to as aryl hydrocarbon hydroxylase (AHH)‡ activity[1, 2]. This enzyme system is of great current interest due to its importance in the activation of chemical carcinogens[3–5]. The AHH activity is usually assayed by a fluorometric method using benzo[a]pyrene (BP) as a substrate; hence the name BP hydroxylase is also used[6–8]. Although only a portion of the BP metabolites are measured by this method[6–10], it is a rapid, sensitive and useful tool for assaying the AHH activity. The method has been widely used in the fields of chemical carcinogenesis and drug metabolism. Nevertheless, there is great variability in the literature on the AHH activities of comparable samples. The AHH activity obtained in this laboratory[11–15] is usually higher than the values reported by some investigators[9, 10, 16–18]. The differences are probably due to the different assay conditions used, although the strains of animals may also be a factor in affecting the results.

In order to resolve the differences, we have analyzed several aspects of the assay procedure. It was observed that preincubation of the reaction mixture at 37° before the addition of BP could greatly affect the AHH activity. These factors were systematically studied and we have observed the possible existence of high and low catalytic states of the microsomes. Due to its hydrophobicity, the substrate BP is usually added to the reaction mixture either in methanol or acetone. We have observed significant differences when these two solvents were compared, and the results cannot be interpreted with the known effects of organic solvents on the AHH system[16]. The results of a comparative study of the effects of these solvents on the AHH activity are summarized in this report.

EXPERIMENTAL PROCEDURE

Preparation of microsomes. Microsomes were prepared from male Long–Evans rats (body weight 70–125 g) which had received a daily i.p. injection of 3-methylcholanthrene (in corn oil, 25 mg/kg body weight) or phenobarbital (in saline, 75 mg/kg body weight) for 4 days, or no treatment. The livers from 15 to 20 animals were pooled and homogenized in a 0.05 M Tris–HCl buffer, pH 7.4, containing 1.15% KCl. The homogenate was centrifuged at 10,000 *g* for 20 min to remove mitochondria, nuclei and cell debris. The post-mitochondrial supernatant fraction was centrifuged at 105,000 *g* for 90 min. The microsomal pellet was washed once with a solution containing 1.15% KCl and 10 mM EDTA, and resuspended in 0.25 M sucrose. The cytochrome P450 contents (mean ±standard error) of MC, PB and control microsomes were 1.7 ± 0.2, 2.5 ± 0.3 and 0.8 ± 0.1 nmoles per mg of protein respectively. The microsomal suspension was frozen in a dry ice–acetone

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‡The abbreviations used in the text are as follows: AHH, aryl hydrocarbon hydroxylase; BP, benzo[a]pyrene; and MC or PB microsomes, microsomes prepared from 3-methylcholanthrene or phenobarbital pretreated rats respectively.

bath in small portions and stored at −90°. Freshly thawed microsomes were used for the experiments and their AHH activity was found to be not different from the freshly prepared microsomes.

Assays of AHH activity. The assay is similar to the procedure of Nebert and Gelboin[8] but with some modifications[11, 12]. In this paper, details are included to afford comparisons with other versions of the assay[9, 10, 16–18]. Unless otherwise stated, the assay was carried out as follows: The reaction mixture, which contained 100 μmoles potassium phosphate, pH 7.4, 3 μmoles $MgCl_2$, 0.1 μmole EDTA, 0.04 to 0.3 mg of microsomal protein and 0.4 μmole NADPH in a final volume of 1 ml, was preincubated at 37° for 2–3 min and the reaction was initiated by the addition of 80 nmoles BP in 25 μl acetone. After incubation in a shaking water bath at 37° for 5 min, the reaction was stopped by the addition of 4 ml acetone–hexane (1:3, v/v). The mixture was shaken for an additional 10 min at 37°. A 2-ml aliquot of the organic phase was extracted with 4 ml of 1 N NaOH. The fluorescence of the phenolic products in the aqueous phase was measured in a Farrand spectrofluorometer with activation at 396 nm and fluorescence at 522 nm. A standard with a known amount of authentic 3-hydroxybenzo[a]pyrene in the incubation mixture was run in parallel with the assay for calculating the amount of product formed[8]. The spectrofluorometer was standardized each time with quinine sulfate. The assay was carried out in duplicate under dim light. The differences between the duplicates were less than 10 per cent. The hydroxylase activity was expressed in turnover numbers, i.e. nmoles product formed/min/nmole of cytochrome P450.

Other assays. Protein was determined by the method of Lowry *et al.*[19]. Cytochrome P450 concentration was measured with a Cary 17 recording spectrophotometer according to the method of Omura and Sato[20] using an extinction coefficient of 91 $cm^{-1}\,mM^{-1}$ for $A_{450-490\,nm}$.

Chemicals. The sources of chemicals were as follows: benzo[a]pyrene and NADPH from Sigma Chemical Co., St. Louis, MO, 3-methylcholanthrene from Mann Research Laboratories, New York, N.Y., phenobarbital and ethylmorphine · HCl from Merck & Co., Rahway, N.J., and 3-hydroxybenzo[a]pyrene from the National Cancer Institute.

RESULTS

Effects of preincubation on AHH activity. In the AHH assay, the microsomes were usually mixed with the buffer solution and NADPH at a temperature of 20° or lower. The mixture can be preincubated at 37° for 2 min prior to the initiation of the reaction with BP, or the reaction can be initiated without preincubation (Fig. 1). In both cases the assays were carried out in a 37° water bath. With the preincubation, the initial reaction temperature was 37°. Without preincubation, the initial reaction temperature was between 15–20° and the time curve of the reaction intersects at 0.5 or 0.7 min rather than at the origin. The rates calculated from the slopes of the time curves of the control microsomes are 0.80 and 0.32 for the preincubated and non-preincubated systems respectively. The different rates are due to the preincubation process rather than to the differences in the initial reaction temperatures because less than 1 min is required for the temperature to increase from 15–20 to 37°. A similar enhancement effect of preincubation was observed with MC microsomes showing turnover numbers of 2.1 and 1.3 respectively. With the preincubated MC microsomes, there was a slight deviation from linearity, and the number 2.1 represents an average value for the first 5 min.

The sequence of addition of the reagents is important to the enhancement effect (Table 1). The effect was observed when the microsomes were preincubated at 37° for 1–3 min, either in the presence or absence of NADPH. However, the effect was negated if BP was also present in the preincubation mixture. This effect is not due to the acetone in which the BP is dissolved, since preincubation in the presence of an equal aliquot of acetone did not abolish the enhancement. The slight reduction in activity (Expt. 5 vs Expt. 2, Table 1) is probably due to the inhibitory action of acetone. A similar enhancement effect was also observed with methanol as the solvent for BP (results not shown). Cooling of the preincubated reaction mixture in ice for 1 min (to *ca.* 10°) nullified the preincubation, a conclusion drawn by a comparison of the results of Expts. 6 and 2. The experiments also serve to examine whether the preincubation enhancement is due to the formation of active intermediates (such as hydroperoxides) or the decomposition of endogenous inhibitors during the preincubation period. Since the effects of these factors are not expected to be eliminated by chilling the reaction mixture for 1 min, the results of Expts. 6 and 7 indicate that these factors are not responsible for the preincubation enhancement.

The extent of the preincubation enhancement

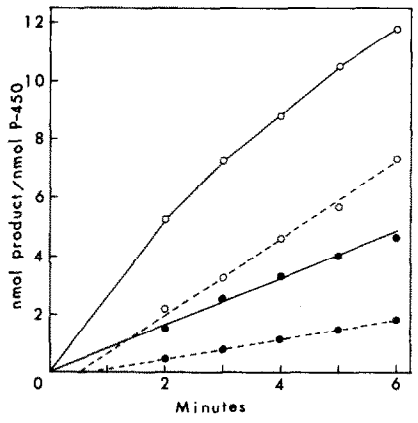


Fig. 1. Effect of preincubation on the rate of benzo[a]pyrene hydroxylation. The assay mixture contained control microsomes (●) or MC microsomes (○) corresponding to 0.24 or 0.19 nmole cytochrome P-450 respectively. They were preincubated for 2 min at 37° (——) or without preincubation (----) before the reaction was initiated by the addition of 80 nmoles BP in 25 μl acetone.

Table 1. Effects of preincubation and the sequence of reagent addition on aryl hydrocarbon hydroxylase activity*

Experiment	Additions to preincubation mixture	Preincubation (min)	Initiation of the reaction with	Activity (turnover number)
1		0	BP, NADPH	0.39
2		2	BP, NADPH	0.86
3	NADPH	2	BP	0.91
4	BP	2	NADPH	0.37
5	Acetone (25 μl)	2	BP, NADPH	0.77
6†		2 min, then chill to 10° in 1 min	BP, NADPH	0.38
7	NADPH	2 min, then chill to 10° in 1 min	BP	0.36

* The basic reaction mixture, 1 ml, contained control microsomes corresponding to 0.3 nmole cytochrome P-450, 0.4 μmole NADPH and 80 nmoles BP added in 25 μl acetone. The reactions were carried out at 37° for 5 min.

† The chilled reaction mixture was preincubated again for 2 min to bring the temperature to 37° before the initiation of the reaction.

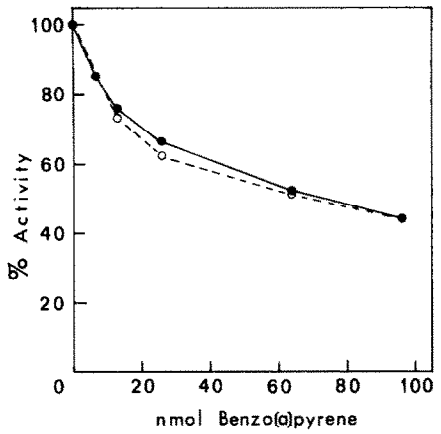


Fig. 2. Aryl hydrocarbon hydroxylase activity as affected by the benzo[a]pyrene concentration in the preincubation mixture. The reaction mixture, containing control microsomes (●——●) or MC microsomes (○– – –○) corresponding to 0.13 mg protein and the indicated amount of BP, was preincubated at 37° for 2–3 min. Additional BP was added as required to give a final concentration of 96 μM, and the reaction was initiated immediately by 0.4 μmole NADPH.

was affected by the concentration of BP in the preincubation mixture (Fig. 2). The enhancement was almost completely eliminated by 96 μM BP and was reduced to half by approximately 15 μM BP. This was observed when either MC or control microsomes were used in the experiment.

Effect of temperature on the preincubation enhancement. In this study, we took advantage of the fact that no enhancement was observed if the microsomes were preincubated in the presence of BP. The reaction mixtures were preincubated in the absence and presence of 80 nmoles BP, and the results were expressed as an activity ratio (Table 2). An activity ratio larger than 1 indicated a preincubation enhancement. It is seen that the enhancement was observed when the microsomal suspension was preincubated at 25° or higher temperatures prior to the addition of BP, but it was not observed when the preincubation was carried out at 20° or below.

Nature of the preincubation enhancement. The above observations may be due to either of two possibilities: (1) the binding with BP at temperatures above 25° enables the microsomes to exist in a high activity state, whereas binding at 20° or below would limit the microsomes to a low activity state; and (2) the optimal binding of BP to the microsomes takes place at 25–37° but not at 20° or below; hence the effective substrate concentration of BP is lower when BP is added to microsomes at the lower temperatures. If the latter were true, preincubating the microsomes in the presence of BP and then initiating the reaction at 37° with more BP and NADPH should manifest the preincubation enhancement effect. However, this was not observed (Expt. 3, Table 3). The results tend to rule out the second and favor the first possibility. The preincubation enhancement was also partially abolished by 80 nmoles of 3-methylcholanthrene (Expts. 6 and 7, Table 3). Ethylmorphine, an *N*-demethylase substrate of the mono-oxygenase system, inhibited the AHH reaction in general but did not negate the pre-incubation effect (Expts. 8 and 9, Table 3).

Effect of acetone and methanol on the AHH assay. While acetone has been reported to be inhibitory to the AHH activity[16], we have always obtained higher activities with control and PB microsomes when acetone rather than methanol was used as the solvent for BP. A comparative study using these two solvents has been done with different types of microsomes, and one set of experiments is shown in Table 4. When acetone was used as the solvent, the AHH activities of control and PB microsomes were higher than when methanol was used, but the reverse was observed with MC microsomes. Such differences between these two solvents have been consistently observed in studies which consisted of more than 300 determinations with different preparations of microsomes.

Table 2. Effect of temperature on the preincubation enhancement*

Expt.	Preincubation temperature	Assay temperature	Activity ratio
1	20°	37°	1
2	30°	37°	1.7
3	37°	37°	2.0
4	30°	30°	1.5
5	25°	25°	1.8
6	18°	18°	1
7	14°	14°	1

* Assay conditions were similar to those in Table 1. The microsomes were preincubated (a) in the presence and (b) in the absence of 80 nmoles BP. The ratio of the activities of (b) over (a) is referred to as the activity ratio. A ratio larger than 1 indicates the preincubation enhancement.

Table 3. Effects of substrates on the preincubation enhancement*

Expt.	Preincubated in the presence of	Preincubation (min)	Activity (turnover number)
1†		0	0.32
2†		2	0.72
3†	BP (80 nmoles)	2	0.31
4		0	0.33
5		2	0.83
6	MC(80 nmoles)	0	0.30
7	MC(80 nmoles)	2	0.42
8	EM(5 μmoles)	0	0.13
9	EM(5 μmoles)	2	0.28

* Assay conditions were similar to those in Table 1, except that an extra 80 nmoles BP, 80 nmoles MC (3-methylcholanthrene) or 5 μmoles EM (ethylmorphine) was also present in some of the preincubation mixtures. The reactions were initiated by the addition of 80 nmoles BP (in 25 μl acetone) and NADPH.

† Experiments 1 and 2 were initiated with 160 nmoles BP (in 50 μl acetone) and NADPH. The total amount of BP in Expt. 3 was 160 nmoles.

Table 4. Effect of acetone and methanol on microsomal aryl hydrocarbon hydroxylase activity*

Microsomes	Solvent for benzo[a]pyrene: Acetone	Methanol
Control	0.88 ± 0.02 (9)	0.45 ± 0.04 (8)
PB	0.53 ± 0.04 (6)	0.29 ± 0.04 (6)
MC	3.00 ± 0.09 (9)	3.56 ± 0.22 (8)

* Results are expressed as turnover numbers in the form of mean ±standard error (number of determinations). The reaction mixture contained control or PB microsomes corresponding to 0.13 nmole cytochrome P-450, or MC microsomes corresponding to 0.06 nmole cytochrome P-450. The assay was carried out according to the standard procedure described in Experimental Procedure, with BP added either in 50 μl acetone or 50 μl methanol.

The effects of these solvents were further studied by adding them to the basic assay system, which already contained either 25 μl acetone or 50 μl methanol (Fig. 3). With control microsomes and with either acetone or methanol as the solvent for BP, the AHH activity was inhibited to a lesser extent by 50 μl acetone than methanol, although both solvents inhibited the activity by a similar extent when 100 μl was added. It can be estimated by extrapolation that the inhibition caused by the initial 25 or 50 μl of solvent acetone (0.34 or 0.68 M) was 7 or 15 per cent, respectively, for both control and MC microsomes. The initial 50 μl methanol (1.23 M) caused a 30 per cent inhibition with control microsomes, but a 13 per cent stimulation with MC microsomes. The present results are consistent with those of Wiebel *et al.*[16] in that, at a concentration of 5%, acetone but not methanol inhibits the AHH activity of MC microsomes. The results are in disagreement with theirs[16], however, in that we have observed the inhibition of AHH activity of control microsomes by both acetone and methanol. The reason for such a discrepancy is not apparent. Methanol can be metabolized by the mono-oxygenase system of control microsomes at a rate of 8 nmoles/min/mg of protein, which is similar to the rate of demethylation of ethylmorphine (C. S. Yang, unpublished observations). It is conceivable that the inhibitory action of methanol is at least partially due to its competition with BP for the mono-oxygenase system.

DISCUSSION

The present results demonstrate that the AHH activity can be greatly affected by two factors of the assay, namely preincubation and the solvent for BP. The enhancement effect of preincubation can explain the differences between the presently observed AHH values (Table 4) and those reported by Holder *et al.*[9]. Thus, without preincubation these authors obtained turnover numbers of 0.36, 0.31 and 1.34 for control, PB and MC microsomes, respectively, rather than 0.88, 0.53

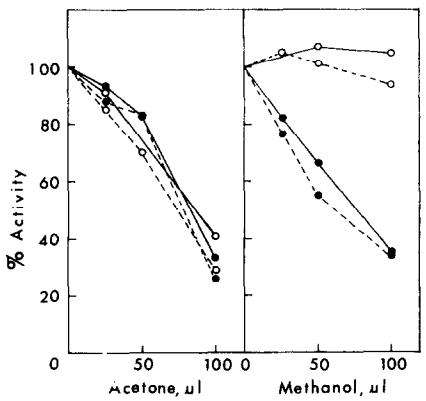
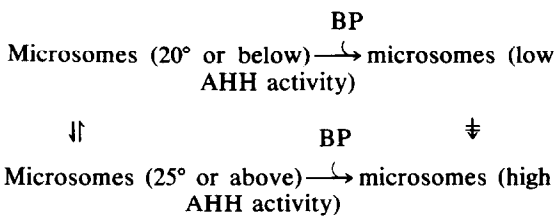


Fig. 3. Effects of acetone and methanol on microsomal aryl hydrocarbon hydroxylase activities. The assay mixture contained control microsomes (●) or MC microsomes (○) corresponding to 0.15 to 0.31 or 0.13 nmole cytochrome P-450, respectively, and the indicated amounts of acetone and methanol. BP was then added either in 25 μl acetone (——) or 50 μl methanol (----).

and 3.00, which were obtained with preincubation in our laboratory.*

The molecular basis of the preincubation enhancement is not very well understood. The observations are consistent with the following scheme:



Binding of BP at temperatures below 20° would limit the microsomes to the low activity state, which is not converted to the high activity state when assayed at 37°, whereas binding at 25° or above would allow the microsomes to be in the high activity state. This can be caused by a temperature-dependent binding of BP to (a) the cytochrome P-450 molecules directly, or (b) the phospholipids in the microsomal membrane. The spin state of cytochrome P-450 is known to be affected by temperature[21]. It is possible that preincubation of microsomes with BP at different temperatures could affect the spin state and the catalytic activities of cytochrome P-450. On the other hand, there is no evidence that this enzyme can exist in either a high or low activity conformation. The amount of BP in the preincubation mixture that eliminates 50 per cent of the enhancement, i.e. 15 μM as calculated from Fig. 2, does not correlate with K_m values of 0.3 to 2.4 and 1.0 to 8.6 μM of BP for MC and control microsomes respectively[22–24]. On the other hand, a temperature-dependent distribution of membrane components in microsomes has been reported[25]. Aromatic hydrocarbons or steroids at the quantities used in this study, i.e. 20–80 nmoles BP/0.15 mg of microsomal lipids, are known to affect the structure of membrane lipids[26–28]. It is possible that the difference in membrane structure may be responsible for the high and low catalytic states of the microsomes.

The solubility of BP in water has been reported to be between 9 and 24 nM[29]. Most of the BP added to an assay mixture is bound to microsomes (C. S. Yang, unpublished observations). It is possible that the effective substrate concentration reflects the BP molecules bound to microsomal membrane rather than those in aqueous solution. The results obtained with acetone and methanol as solvents for BP are understandable if we assume that the former solvent enables BP to be at a higher effective concentration than the latter. In addition, acetone is less inhibitory than methanol at the level used, i.e. 25 or 50 μl. MC microsomes have a lower K_m for BP[22–24], and may operate at maximal velocity even with the lower effective BP concentration provided by methanol. This, plus the stimulatory effect of methanol on the AHH activity of MC microsomes, may account for the higher activity of MC microsomes with methanol than with acetone.

Although the solvent effect on the microsomal AHH activity is not understood at the detailed molecular level, the present results clearly demonstrated the superiority of acetone over methanol as the solvent for BP in the AHH assay. First, lower concentrations of acetone can be used.† Second, acetone inhibits the AHH of different types of microsomes uniformly (approx 7 per cent at 0.34 M), whereas methanol inhibits the AHH activity of control microsomes but stimulates the activity of MC microsomes. Third, the results are less reproducible with methanol as the solvent. The second point is especially important when dealing with the induction of AHH activities. For example, the data in Table 4 indicate that the cytochrome P-448 in MC microsomes is three to four times as active as the cytochrome P-450 in control microsomes when assayed with the acetone system, but is eight times as active with methanol.

* This point has been discussed with Drs. A. Y. H. Lu, S. West and W. Levin. Their AHH assay method is essentially the same as ours, except for the preincubation step. These investigators have also observed the preincubation enhancement with the reconstituted AHH system.

†After shaking benzo[a]pyrene (in excess) with acetone or methanol at 20° for 15 hr, solutions of 7.84 or 2.71 mM, respectively, can be made.

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