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BENZPYRENE PRETREATMENT CHANGES THE KINETICS AND pH OPTIMUM FOR ANILINE HYDROXYLATION *IN VITRO*, BUT NOT THOSE FOR BENZPHETAMINE DEMETHYLATION *IN VITRO* BY RAT LIVER MICROSOMES*

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Abstract—The effects of benzpyrene pretreatment on aniline *para*-hydroxylation, benzphetamine demethylation and benzpyrene hydroxylation in rat liver microsomes were studied and the following results were obtained:

The pH optimum for the metabolism *in vitro* of aniline is shifted from pH 7·0 to pH 8·1 by benzpyrene pretreatment. When the spectral dissociation constants (K_s) with aniline are compared at pH 7·0 and pH 8·1 using microsomes from benzpyrene-pretreated rats, a pH dependency of K_s is seen. The K_s values for microsomes from control rats are the same at both pH's, but they are different from both the K_s value at pH 7·0 for microsomes from benzpyrene-pretreated rats and the K_s value at pH 8·1 for microsomes from benzpyrene-pretreated rats. K_m values for aniline hydroxylase are altered by changes in pH with hepatic microsomes from either control or benzpyrene-pretreated rats.

Neither the K_m nor the pH optimum for benzphetamine demethylase is changed by benzpyrene pretreatment. The K_s for benzphetamine is also unchanged by pretreatment with benzpyrene.

The K_m value for benzpyrene hydroxylase is changed by pretreatment with benzpyrene, but the pH optimum for the metabolism *in vitro* of benzpyrene does not seem to be altered by benzpyrene pretreatment.

THE POLYCYCLIC hydrocarbons are not only fairly specific in the enzymes of the hepatic microsomal enzyme system which they stimulate,[1] but they also seem to alter the enzyme which is produced. For instance, the polycyclic hydrocarbon, 3-methylcholanthrene, changes the ratio of the 430:455 mμ peaks resulting from the addition of ethylisocyanide to microsomes in suspension.[2] The polycyclic hydrocarbons also alter the carbon monoxide difference spectrum of reduced P450, causing a shift of the major peak from 450 mμ to 448 mμ.[3, 4]

The experiments described in this paper were designed after we noticed during optimization of conditions for several drug metabolism assays *in vitro*, that benzpyrene (BP) pretreatment caused a shift in the pH optimum for the *para*-hydroxylation of aniline. We have attempted to show that the hepatic enzyme system catalyzing aniline

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para-hydroxylation is different in control rats from that in rats pretreated with BP, and that this is not the case with a system such as benzphetamine demethylase which is not stimulated by BP. The metabolism of BP itself was also studied because it appears that the BP hydroxylase system is affected by pretreatment with BP in a third way that is different from the way in which either aniline hydroxylase or benzphetamine demethylase is affected.

METHODS

Male Sprague–Dawley rats weighing between 240 and 260 g were used in all experiments described in this paper. The animals were allowed free access to Wayne Lab Blox and water from the time of arrival until sacrifice and were allowed 3–4 days to acclimate to our animal quarters before treatments were begun. Rats receive either 3,4 benzpyrene (BP) dissolved in corn oil (45 mg/kg), or an equal volume of corn oil alone 72 hr prior to sacrifice. The animals were killed between 6:30 and 7:00 a.m. They were stunned by a blow on the head and decapitated. Livers were quickly removed and placed in 1·15% KCl on ice. Three livers from each pretreatment were pooled for each experiment. All remaining procedures were carried out at 0–4° unless otherwise indicated. After blotting, the livers were weighed and put back into KCl solution. They were then minced with scissors and the 1·15% KCl solution was poured off through a layer of gauze. After repeating this mincing and rinsing procedure once, a 33% homogenate of the livers in 1·15% KCl was prepared with a Potter–Elvehjem homogenizer having a plastic pestle.

The homogenate was centrifuged at 9000 *g* for 20 min. The microsomal pellet was obtained by centrifugation of the 9000 *g* supernatant in either a Beckman L2-65B or an IEC B-60 ultracentrifuge for 100,000 *g*-hr (e.g. 105,000 *g* for 57 min or 226,000 *g* for 27 min). The pellet was resuspended by homogenizing in 1·15% KCl and resedimented. The washed microsomal pellet was resuspended in 1·15% KCl to a volume equal to half that of the 9000 *g* supernatant and microsomal protein content was estimated by the method of Lowry *et al.*[5] Crystalline bovine serum albumin served as the standard. The microsomal suspension was then adjusted to 15 mg microsomal protein per ml with 1·15% KCl.

Aliquots of the above microsomal suspension were used for the metabolism studies *in vitro*. Necessary dilutions were made in 0·1 M Tris buffer of the desired pH for the determination of the spectral dissociation constant (K_s) for various substrates. For measurement of the aniline K_s, dilutions were made to 3·0, 2·0 and 1·0 mg of microsomal protein per ml of the final cuvette contents. Since no significant regression[6] of a plot K_s vs. mg microsomal protein per ml was observed over this range of protein concentrations, these dilutions were counted as being triplicate measurements in any given experiment. Because of the smaller spectral change per mg microsomal protein per ml given by benzphetamine, it was necessary to use dilutions of 3·0, 2·5 and 2·0 mg microsomal protein per ml of final cuvette contents. The range of protein concentrations studied here did not give a significant regression when plotted as above, so again the 3 protein concentrations were considered as triplicate measurements.

The incubation mixture used to estimate the amount of aniline *para*-hydroxylated by the microsomal enzyme system contained 0·3 m-mole Tris buffer at the desired pH, 12·5 μmoles NADP (Sigma Chemical Co.), 0·25 ml of 100,000 *g* supernatant fraction from normal, control rats for the generation of NADPH, 3·75 mg microsomal

protein, 10·0 μmoles aniline HCl, 10 μmoles of glucose 6-phosphate and water, when necessary, to make the final volume of the mixture 2·5 ml. Unless otherwise noted, nicotinamide was not added to any incubation mixtures. One marble was added to each beaker (15 ml capacity) of incubation mixture to insure adequate agitation, and the incubation was carried out under an atmosphere of O_2 at 37° for 30 min in a Dubnoff incubator with a shaking rate of 100 rpm. When the apparent K_m or V_{max} was to be determined, the duration of incubation was 15 min. The extent of aniline *para*-hydroxylation under these conditions was estimated by the method of Kato and Gillette[7] as modified by Gram *et al.*,[8] which measures the amount of *para*-aminophenol formed.

The conditions for the estimation of benzphetamine demethylation were the same as for aniline hydroxylation except that 1·8 μmoles NADP was added, the aniline was replaced by 3·0 μmoles (+) benzphetamine, and 10·0 μmoles of semicarbazide HCl was also added. The method of Cochin and Axelrod was used to measure the formaldehyde formed by the demethylation of benzphetamine.[9]

Ring hydroxylation of BP was estimated by the method of Wattenberg *et al.*,[10] which measures the amount of 8-hydroxybenzpyrene (8-OH BP) formed. We modified this method in only one way. We did not store the incubation mixture–acetone mix overnight in the cold. Instead we proceeded directly with extraction into skellysolve B. Since we were unable to obtain a sample of 8-OHBP, quinine sulfate was used as the standard, and the fluorescence of a known concentration of quinine sulfate was used to calculate mμmoles 8-OHBP formed. According to Dr. Wattenberg, 0·036 mμmole 8-OHBP/ml in 1 N NaOH gives a fluorescence which is equal to that given by 0·3 μg quinine sulfate per ml in 0·1 N H_2SO_4 at an excitation wavelength of 400 mμ and an emission wavelength of 522 mμ. The incubation mixture contained 0·3 m-mole TRIS buffer of the desired pH, 10·0 μmoles glucose 6-phosphate, 2·9 μmoles NADP, 0·2 ml 100,000 *g* supernatant from control animals for NADPH production, 1·5 mg microsomal protein, 1·6 μmoles BP, and water, when necessary, to make the final volume 2·5 ml. Conditions of incubation time, temperature, etc. were as described for aniline hydroxylation. Nicotinamide was not added to any incubation mixture wherein benzpyrene metabolism was being studied.

The metabolism *in vitro* of the three substrates mentioned above was linear for at least 30 min, the amounts of protein used were within the range of linearity, and the amounts of cofactors added were found to be optimal (unpublished observations).

The spectral dissociation constant, K_s, was measured by the method of Remmer *et al.*,[11] with the slight modification that the absorbance was measured from the peak to the trough of the spectrum rather than from an isosbestic point. This absorbance between the trough at 395 mμ and the peak at 435 mμ for aniline and between the peak at 390 mμ and the trough at 420 mμ for benzphetamine was designated ΔA. Measurements of ΔA were performed on a Shimadzu MPS-50L recording, double-beam spectrophotometer.

K_s and K_m values were determined by means of Woolf[12] plots ($[s]/v$ vs. [s] or $[s]/\Delta A$ vs. [s]). Best fitting lines were drawn by the method of least squares, and statistical analyses were done by an analysis of variance.[13] $P \leqslant 0{\cdot}05$ was the designated significance level.

The range of substrate concentrations used for the determination of K_s and K_m values is given in the appropriate tables. It should be pointed out here that the K_m and

K_s values so obtained may be valid for only those ranges of substrate and microsomal protein concentrations used, and that the conclusions drawn from the data should not be extended without care to other substrate or protein concentrations.

RESULTS

Figures 1, 2 and 3 show the effects of pH on the hepatic microsomal metabolism *in vitro* of aniline, benzphetamine, and benzpyrene respectively. The pH optimum for

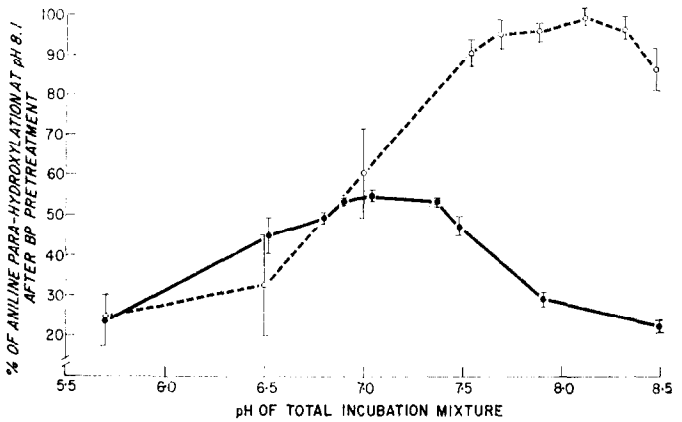

FIG. 1. pH optima for aniline *para*-hydroxylation in control (●—●) and BP-pretreated (○---○) rats. Conditions as given in Methods. Data are normalized so that each point is expressed as a percentage of the rate of *para*-hydroxylation of aniline obtained with microsomes from BP-pretreated rats at pH 8·1. This rate was 36·8 mμmoles per mg microsomal protein per 30 min ± 3·2 (mean ± S.E.). Each point is a mean of the percentages of at least 3 experiments ± S.E. The mean of the pH measured in the incubation mixture is given as a point without any indication of variability for clarity. However, no final pH varied from the point on the graph by more than 0·1 pH unit from experiment to experiment.

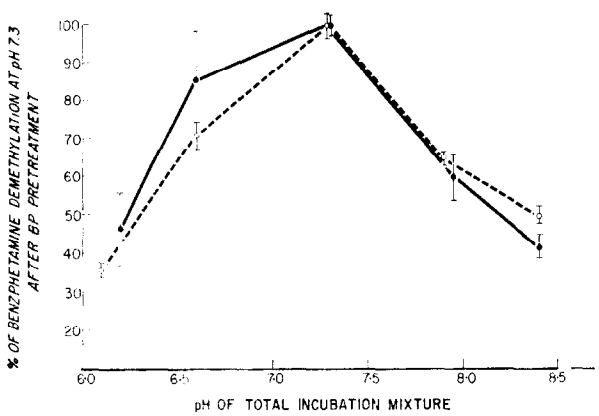

FIG. 2. pH optima for benzphetamine demethylation in control (●—●) and BP-pretreated (○---○) rats. Conditions as given in Methods. Data normalized as in Fig. 1 except that the rate of metabolism obtained with microsomes from BP-pretreated rats at pH 7·3 was taken as 100 per cent. This rate was 180·7 mμmoles per mg microsomal protein per 30 min ± 16·4 (mean ± S.E.). Points are means ± S.E. Variation in pH as in Fig. 1.

metabolism *in vitro* was not shifted after BP pretreatment when the substrate was benzphetamine or benzpyrene. However, when the substrate was aniline, the pH optimum shifted from about pH 7·0 for hepatic microsomes from control rats to about pH 8·1 for hepatic microsomes from rats pretreated with BP. While the peaks were rather broad, the pH optima for further studies of aniline hydroxylation were chosen as pH 7·0 for experiments with microsomes from control rats and pH 8·1 for experiments with microsomes from BP-pretreated rats. These pH's were chosen because they were near the middle of the peaks and gave consistently higher rates of aniline metabolism than other nearby pH's.

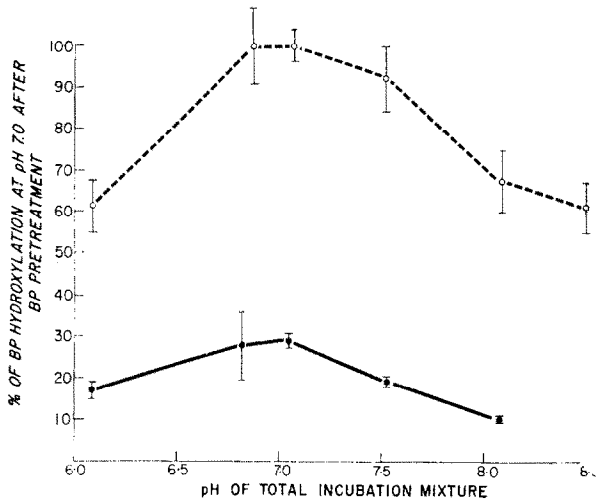

FIG. 3. pH optima for benzpyrene hydroxylation in control (●—●) and BP-pretreated (○---○) rats. Conditions as in Methods. Data normalized as in Fig. 1 except that the activity obtained with microsomes from BP-pretreated rats at pH 7·07 was taken as 100 per cent. This rate of metabolism was 14 4 mμmoles per mg microsomal protein per 30 min ± 3·2 (mean ± S.E.). Points are means ± S.E. Variation in pH as in Fig. 1.

An interesting effect seen in Fig. 1 was that if aniline metabolism was determined at pH 7·0 (or less) no increase in metabolism would be noted after BP pretreatment.

Since the shift in pH optimum might indicate that pretreatment with BP altered aniline hydroxylase qualitatively, we decided to study several other parameters which might reflect a qualitative change in the enzyme system.

As two of our substrates were water-soluble and gave characteristic difference spectra when added to a microsomal suspension, one of the parameters studied was the spectral dissociation constant, K_s. Table 1 gives the K_s values for the Type I substrate, benzphetamine. The values given are for a suspension of microsomes in 0·1 M Tris buffer at a final pH of 7·3. BP pretreatment did not alter the K_s for benzphetamine, but the ΔA_{max} obtained with microsomes from BP-treated rats was about 0·6 that obtained with microsomes from control rats. The ΔA_{max} obtained with microsomes from BP-treated rats could be raised to nearly the control level by the

addition of bovine serum albumin to the cuvette (see Table 1), indicating that residual BP or its metabolites might interfere with the benzphetamine spectrum.

The K_s values for aniline (Type II substrate) are given in Table 2; the ΔA_{max} values given for aniline with microsomes from either control or BP-pretreated rats were not appreciably affected by the addition of albumin. In Table 2, hepatic microsomes from control and BP-pretreated rats were used at both pH 7·0 and pH 8·1. The K_s for control microsomes was slightly lower at pH 8·1; however, this decrease was not statistically significant. When microsomes from rats pretreated with BP were subjected

TABLE 1. K_s AND ΔA_{max} FOR BENZPHETAMINE INTERACTION WITH HEPATIC MICROSOMES FROM CORN OIL OR BENZPYRENE-PRETREATED RATS*

	K_s†		ΔA_{max}‡	
Pretreatment	Without added albumin	With added albumin	Without added albumin	With added albumin
Corn oil	0·015 ± 0·001	0·015 ± 0·002	0·032 ± 0·001	0·030 ± 0·001
BP (45 mg/kg)	0·013 ± 0·002	0·016 ± 0·001	0·019 ± 0·001§	0·027 ± 0·002

* Substrate range was 0·016 mM to 0·166 mM. Concentration of albumin, where added, was 3 mg per ml final cuvette contents.
† Units are mM (mean ± S.E.).
‡ Units are absorbance units per mg protein per ml (mean ± S.E.).
§ Significantly different from control, $P < 0·05$. Means represent three experiments.

TABLE 2. K_s AND ΔA_{max} FOR ANILINE INTERACTIONS WITH HEPATIC MICROSOMES FROM CORN OIL OR BENZPYRENE-PRETREATED RATS*

Pretreatment	Corn oil		Benzpyrene (45 mg/kg)	
pH of final cuvette contents	7·0	8·1	7·0	8·1
K_s† without added albumin	1·31 ± 0·36	1·00 ± 0·16	0·60 ± 0·05‡	1·83 ± 0·03‡
K_s† with added albumin	1·24 ± 0·30	1·15 ± 0·18	0·59 ± 0·02‡	1·92 ± 0·02‡
ΔA_{max}§ without added albumin	0·028 ± 0·009	0·020 ± 0·012	0·046 ± 0·002‖	0·049 ± 0·001‖
ΔA_{max}§ with added albumin	0·030 ± 0·005	0·019 ± 0·008	0·048 ± 0·001‖	0·049 ± 0·003‖

* Each mean represents at least five experiments. Substrate concentration range was 1·0 mM to 10·0 mM. Concentration of albumin, where added, was 3 mg per ml final cuvette contents.
† Units are mM (mean ± S.E.).
‡ Significantly different from the three other K_s means given in this table, $P < 0·05$.
§ Units are absorbance units per mg protein per ml (mean ± S.E.).
‖ Significantly different from corresponding corn oil controls, $P < 0·05$.

to the same conditions, there was a rather striking change in the K_s as the pH was changed. Here the K_s value obtained at pH 7·0 was statistically different from that obtained at pH 8·1, and at both pH's, the K_s values were different from control. The ΔA_{max} values were not statistically different at pH 7·0 from pH 8·1 after BP pretreatment, but were significantly increased when preparations from control rats were compared with preparations from BP-pretreated rats at either pH.

Another way by which a qualitative change in an enzyme might be detected is by

measurement of its apparent K_m. Table 3 gives the apparent K_m values for aniline *para*-hydroxylation, benzphetamine demethylation, and benzpyrene hydroxylation by the hepatic microsomal enzyme system. The apparent K_m for benzpyrene hydroxylation was increased by approximately two and one-half times, and the V_{max} was tripled after BP pretreatment.

As was the case for K_s, K_m for benzphetamine was unchanged by BP pretreatment. The V_{max} was also unaffected.

For aniline hydroxylase, the effects of BP pretreatment and pH on K_m were different from those on K_s. The K_m for the aniline hydroxylase system was unaffected by BP pretreatment alone, but when a comparison was made for the system from control or

TABLE 3. EFFECT OF BP PRETREATMENT AND pH ON THE APPARENT K_m AND V_{max} FOR ANILINE HYDROXYLASE, BENZPHETAMINE DEMETHYLASE, AND BENZPYRENE HYDROXYLASE*

Substrate	Pretreatment	pH	K_m (mM, mean ± S.E.)	V_{max} (mμmoles per mg protein per 15 min, mean ± S.E.)
Aniline	Corn oil	7·0	0·20 ± 0·03	14·0 ± 0·1
(0·25 to 4·00)†	BP (45 mg/kg)	7·0	0·23 ± 0·05	16·0 ± 0·1
	Corn oil	8·1	0·57 ± 0·05‡	11·0 ± 0·1
	BP (45 mg/kg)	8·1	0·52 ± 0·04‡	21·0 ± 0·1‡§
Benzphetamine	Corn oil	7·3	0·12 ± 0·04	148 ± 22
(0·04 to 2·3)†	BP (45 mg/kg)	7·3	0·12 ± 0·03	150 ± 36
Benzpyrene	Corn oil	7·0	0·023 ± 0·004	2·12 ± 0·52
(0·01 to 0·63)†	BP (45 mg/kg)	7·0	0·052 ± 0·009§	6·86 ± 2·05§

* Each mean represents at least four experiments.
† Range of substrate concentrations used (mM).
‡ Significantly different from both pretreatments at pH 7·0, $P < 0·05$.
§ Significantly different from corn oil control at the same pH, $P < 0·05$.

BP-pretreated rats, the K_m for either preparation was doubled on increasing the pH from 7·0 to 8·1. Although the K_m values were the same before and after BP pretreatment when K_m was measured at any one pH, the V_{max} was changed by BP pretreatment when V_{max} was measured at pH 8·1. At this pH, the K_m values for the control and BP-pretreated systems were the same, but the V_{max} was nearly doubled by BP pretreatment.

DISCUSSION

Wada *et al.*[14] and Anders[15] have proposed, on the basis of prednisolone inhibition[14] or acetone activation[15] of aniline hydroxylase, that there may be two enzymes which are functional in aniline *para*-hydroxylation. The evidence presented in this paper may support such a postulate. The data presented here suggests that one of these two enzymes is preferentially induced or stimulated by BP pretreatment. The enzyme which is induced by BP seems to have a higher pH optimum (Fig. 1) and a higher K_m, if K_m is estimated at the optimal pH, i.e. pH 7·0 for microsomes from control rats and pH 8·1 for microsomes from BP-pretreated rats. It would appear from Table 3 that one can partially discriminate between the activity due to one or the other of the

enzymes by changing the pH. It would seem possible that in control rats aniline hydroxylase activity is a mixture of the activities of a low K_m, low V_{max}, pH 7·0 optimum-enzyme and a high K_m, high V_{max}, pH 8·1 optimum-enzyme in which the activity of the low K_m, low V_{max}, pH 7·0 optimum-enzyme predominates. BP pretreatment causes a relative increase in the high K_m, high V_{max}, pH 8·1 optimum-enzyme, thus increasing the activity of aniline hydroxylase at pH 8·1.

In this paper, the K_s data for aniline are interpreted only to indicate that a qualitative change has been effected in the aniline hydroxylase system by BP pretreatment. The K_s data might be expected to be difficult to explain because, if we are indeed working with an altered enzyme or a combination of a control and an altered enzyme, the extinction coefficients of the aniline–enzyme complexes might be expected to be different in hepatic microsomal preparations from BP-pretreated rats as compared with those from control rats. The aniline–enzyme complex extinction coefficient might also be expected to be different in control and BP-pretreated rats, since aniline is thought to bind to the heme portion of the molecule[16, 17] and polycyclic hydrocarbons are known to alter the absorption spectrum of cytochrome P450.[2, 3]

Another piece of data which points out that changes in K_s and ΔA_{max} cannot be correlated well with changes in K_m and V_{max} (at least at present) is shown in Tables 2 and 3. While K_m, K_s, V_{max}, and the pH optimum of the enzyme system which demethylates benzphetamine were left unchanged by BP pretreatment, the ΔA_{max} was greatly affected. As pointed out earlier, the addition of albumin to the microsomal suspension seemed to increase the ΔA_{max} for microsomes from BP-pretreated rats nearly to control levels. This might indicate that BP or one of its metabolites interferes with the benzphetamine difference spectrum, but not with the metabolism *in vitro* of benzphetamine by hepatic microsomes.

An examination of Table 3 shows that BP affected its own metabolism in a way that was different from the metabolism of either Type I or Type II compounds. While the pH optimum was left unchanged, the K_m was altered and the V_{max} was increased about 3-fold.

Alvares *et al.*[18] and Gurtoo *et al.*[19] have reported that polycyclic hydrocarbon pretreatment caused marked decreases in K_m for benzpyrene hydroxylase activity measured in rat liver preparations. On the other hand, we found that benzpyrene pretreatment caused an increase (doubling) of K_m for benzpyrene hydroxylase using rat liver microsomes (Table 3, bottom 2 lines). We feel the differences between our results and those of Alvares and Gurtoo may be due to two factors: (1) the time between the last dose of polycyclic hydrocarbon inducer, and (2) the range of substrate concentrations used in establishing K_m and V_{max}.

We sacrificed our animals 72 hr after treatment with benzpyrene whereas both Alvares *et al.*[18] and Gurtoo *et al.*[19] sacrificed animals 24 hr after treatment with either benzpyrene [19] or 3-methylcholanthrene.[18] Our range of substrate concentration used was much greater than either Alvares *et al.*[18] or Gurtoo *et al.*[19] and the ranges used by ourselves and these other authors did not overlap. The lowest concentration of benzpyrene that we used was the highest concentration used by Alvares *et al.*[18] or Gurtoo *et al.*[19] Our substrate range overlaps the actual K_m found, whereas with both Alvares *et al.*[18] and Gurtoo *et al.*[19] their calculated K_m is above the highest substrate concentration used by them in their incubations.

These differences in protocol can explain the differences in results if we assume

either of two things: (1) 24 hr are not long enough to get rid of benzpyrene bound to microsomes, and (2) the plot of 1/v vs. 1/S over a wide range of substrate concentrations may actually be a curve concave downwards. In the first case, appreciable "inducers" (relative to substrate added) will be present during kinetic studies done by Alvares *et al.*[18] or Gurtoo *et al.*[19] but not in our studies. This is because we waited longer, thus allowing the inducer to be metabolized before sacrifice (72 hr vs. 24 hr) and we used much higher substrate concentrations in our enzyme assays.

Both factors (longer time between injection of inducer and sacrifice of animals, and use of higher substrate concentration) would favor the actual substrate available to the enzyme being nearly identical to the amount of substrate added in our experiments, whereas these two substrate concentrations would be quite different in the experiments of Alvares *et al.*[18] and Gurtoo *et al.*[19] In these latter cases, added substrate would be much less than actually available for metabolism. The net result would be that our measured K_m would be greater than theirs in the inducer–pretreated animals. The use of 3-methylcholanthrene instead of benzpyrene as inducer would not affect this end result, since 3-methylcholanthrene could saturate nonspecific binding sites on liver microsomes and make the concentration of benzpyrene (substrate) available to the hydroxylase enzyme much higher than in untreated animals.

If the 1/v vs. 1/S plot is actually a curve concave downward over the substrate concentrations used by ourselves and the other 2 groups of workers, then we would be working in the range where the curve would have a greater slope while Alvares *et al.*[18] and Gurtoo *et al.*[19] would be working in the range where the curve has a lesser slope. This also would make our K_m larger than that found by Alvares *et al.*[18] or Gurtoo *et al.*[19] In the case of aniline as substrate we find that plots of 1/v vs. 1/S are not linear, but do have curvature.[20] Thus, we already have evidence with microsomal enzymes, but so far only with other substrates (aniline rather than benzpyrene) that kinetic "constants" may vary with range of substrate concentration used in their determination.

In view of the points raised in this paper it becomes evident that when one is attempting to find optimal conditions for use in metabolism *in vitro*, the conditions for tissue preparations from both control and pretreated animals should be determined, and pH optima should not be overlooked, since evidently "physiological" pH may not always be optimal. Also, any changes in pH optima discovered could be useful in deciphering the changes in enzyme–substrate interaction resulting from enzyme "induction".

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