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The Prediction of Benzo[a]pyrene Clearance by Rat Liver and Lung from Enzyme Kinetic Data

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SUMMARY

The metabolic clearance of circulating benzo[*a*]pyrene (B[*a*]P) by liver and lung of control and 3-methylcholanthrene (3MC)-pretreated rats was predicted according to the perfusion-limited model from apparent enzyme kinetic constants determined in microsomal incubations. These predictions were tested in isolated organs perfused at normal organ flow. From microsomal incubations the apparent enzyme kinetic constants of B[*a*]P metabolism were determined. The apparent K_m of liver microsomes was decreased 100 times by pretreatment with 3MC, while the K_m of lung microsomes remained at about 0.2 μM. Maximal velocity of B[*a*]P metabolism was much greater in microsomes from liver than in those from lung of both control and 3MC-pretreated rats. Liver was found to have a far greater capacity for B[*a*]P metabolism (intrinsic free clearance) than lung. However, this large disparity was not evident in the predicted clearances. Perfused organs had B[*a*]P clearances very close to those predicted from the model. At normal (*in vivo*) organ flows, control rat lung had a clearance of 1.0 ± 0.1 ml/min, whereas liver had a clearance of 5.9 ± 0.2 ml/min. Corresponding clearances in organs from 3 MC-pretreated rats were 8.9 ± 0.5 and 6.7 ± 0.6 ml/min for lung and liver, respectively. Small discrepancies between predicted and observed values could not be explained by non-uniform distribution of B[*a*]P or shunting of flow. These results suggest that enzyme kinetic data can be used to assess accurately the ability of lung and liver to clear xenobiotic compounds such as B[*a*]P and that, despite the great disparity in their metabolic capacity, under certain conditions these two organs may function equally well in the removal of circulating compounds from the blood.

INTRODUCTION

The ability of the body to eliminate highly lipid-soluble xenobiotic compounds depends on its capacity to metabolize those compounds to more water-soluble forms. Almost all organs of the body are capable of performing such transformations; however, little is known of the relative roles these organs play *in vivo*.

Rane *et al.* (1) have proposed a model using enzyme kinetic parameters determined in broken-cell preparations to predict the ability of liver to clear circulating drugs. In this model the capacity of liver to metabolize a compound is expressed as a clearance term, the intrinsic free clearance (Cl'_{int}), which is defined as that volume of liver water cleared of an agent per unit of time. In terms of the enzyme kinetic parameters V_{max} and K_m,

$$Cl'_{int} = V_{max}/K_m \quad (1)$$

Cl'_{int} is thus a constant under first-order conditions and reflects the maximal ability of liver to remove the substrate from the circulation.

The actual ability of an organ to remove a substance from the circulation depends on on other factors as well. A model originally proposed by Rowland *et al.* (2) and further substantiated by the work of others (3, 4) describes this ability in terms of clearance, or that volume of blood perfusing an organ from which the compound is completely and irreversibly removed per unit of time. Clearance (Cl) depends on the flow (Q) to the organ, the fraction of the substance unbound in the blood (f_B), and the intrinsic free clearance (Cl'_{int}):

$$Cl = \frac{Q \cdot f_B \cdot Cl'_{int}}{Q + (f_B \cdot Cl'_{int})} \quad (2)$$

Therefore, if the enzyme kinetic parameters, organ flow, and free fraction in the blood are known, the ability of that organ to remove the compound can be predicted. Several recent studies (1, 5–7) have demonstrated that such predictions are useful for estimating the ability of organs to clear xenobiotic compounds from the circulation.

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B[a]P[2] is a ubiquitous environmental contaminant formed during the incomplete combustion of organic matter. This polycyclic aromatic hydrocarbon is very lipid-soluble and in the absence of metabolism would remain in the body for long periods of time. B[a]P in rats is initially metabolized by the microsomal cytochrome P-448 enzyme(s) system known AHH (8). Oxidation products formed by this process are further metabolized enzymically or degenerate spontaneously to a number of different products. AHH activity occurs in both rat liver and lung and is significantly enhanced by prior exposure of an animal to 3MC (9) or to other agents.

The purpose of this study was to determine the apparent microsomal enzyme kinetic parameters for the metabolism of B[a]P and to predict from them the ability of intact liver and lung of control and 3MC-pretreated rats to clear B[a]P from the circulation. Subsequently, this prediction was tested by examining the clearance of B[a]P in isolated livers and lungs perfused at normal organ flow.

METHODS

Animals. Male Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, Mich.) weighing 200–300 g were used in these studies. The animals were housed in plastic cages on corncob bedding in a 12-h light/dark cycle. Food (Wayne Lab-Blox, Allied Mills, Chicago, Ill.) and tap water were allowed ad libitum.

Treatment. 3MC (Pfaltz and Bauer, Inc., Stamford, Conn.) was suspended in corn oil (Mazola, Best Foods, Englewood Cliffs, N. J.) to a final concentration of 10 mg/ml. Each 3MC-pretreated rat received an injection (20 mg/kg, i.p.) of this solution 24 and 48 hr prior to sacrifice. Control rats received an equal volume of corn oil.

Preparation of [^{3}H]B[a]P. [G-^{3}H]-B[a]P, specific activity 17.4 Ci/mmole (Amersham, Inc., Arlington Heights, Ill.), was purified by the procedure of Van Cantfort *et al.* (10) and mixed with non-radioactively labeled B[a]P (more than 99% pure, Aldrich Chemical Company, Milwaukee, Wisc.) to the required concentration and specific activity in either acetone or methanol for the broken-cell assays or perfusions, respectively. These solutions were stored under nitrogen at −20° in the dark until use. Selected solutions were analyzed by high-performance liquid chromatography (11) to confirm nominal concentrations.

Preparation of microsomes. Rats were killed by decapitation, and their livers and lungs were removed and immediately placed in ice-cold 0.05 M Tris-HCl buffer (pH 7.4) containing 1.15% KCl. The organs were then blotted, weighed, and placed in fresh buffer (2 volumes for liver, 4 volumes for lungs). The organs were homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, N. Y.) for 30–45 sec at a setting of 5.5–6.0; the homogenate was centrifuged at 10,000 × *g* for 10 min at 4°. After centrifugation the lipid was aspirated from the supernatant fraction, which was recentrifuged at 100,000 × *g* for 60 min at 4°. The resultant supernatant fraction was decanted and discarded, and the pellet was resuspended in 0.05 M Tris-HCl (pH 7.4 for livers and pH 8.0 for lungs) containing 0.25 M sucrose and 10 mM EDTA to a final volume of 1 ml per initial gram of liver and 2 ml per initial gram of lung. Five microliters per gram of initial weight of an absolute ethanol solution containing 2% (w/v) butylated hydroxytoluene (Sigma Chemical Company, St. Louis, Mo.) were added, and the resuspended pellet was homogenized with six passes of a Teflon-glass tissue grinder. Appropriate dilutions of these preparations were prepared in Tris-HCl buffer (pH 7.4 for liver, pH 8.0 for lung) prior to determination of metabolic activity.

Determination of microsomal AHH activity. The ability of microsomal preparations to metabolize B[a]P was determined in duplicate according to the method of Van Cantfort *et al.* (10). Incubations were carried out at 37° in a shaking incubator in screw-capped tubes (16 × 100 mm). The incubation medium (0.5 ml) consisted of 0.05 M Tris-HCl buffer (pH 7.4 for liver, pH 8.0 for lung) containing bovine serum albumin (1 mg/ml) (Fraction V, Schwarz-Mann, Orangeburg, N. Y.), 3 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM NADPH (reduced form), and an appropriate concentration of microsomal protein as determined in preliminary experiments (see Table 1). In other preliminary experiments, varying the pH between 7.2 and 8.0 did not change AHH activity in either liver or lung microsomal preparations. Reaction was initiated following a 2-min preincubation at 37° by the addition of B[a]P (10 μCi/ml at various B[a]P concentrations) in 20 μl of acetone. Incubations were continued for the appropriate time and stopped by the addition of 1.0 ml of ice-cold 0.15 N KOH in 85% dimethyl sulfoxide.

Extraction of unreacted B[a]P was accomplished by the addition of 5 ml of hexane (two times). Following 10 min of shaking, the layers were allowed to separate and the hexane was aspirated off. High-pressure liquid chromatography of the hexane showed that less than 3% of the B[a]P metabolites was extracted into the hexane phase. Samples (0.5 ml) of the remaining aqueous phase were removed, placed in scintillation vials, and neutralized by the addition of 0.5 ml of 0.15 N HCl. Fifteen milliliters of ACS (Amersham, Inc., Arlington Heights, Ill.) were added and the radioactivity of each vial was quantified by liquid scintillation spectrometry. External standard quench correction was used to convert the raw counts to disintegrations per minute.

Calculation of metabolic activity. The amount (nanomoles) of B[a]P metabolites (A) present in each sample vial was calculated according to the following equation:

$$A = \frac{D_S - D_B}{D_T} \times \text{nmol B[a]P added to the assay mixture} \qquad (3)$$

where D_S is disintegrations per minute in a sample vial, D_B is disintegration per minute in a sample blank vial (identical with an activity vial, but incubated in the absence of NADPH), and D_T is disintegrations per minute in a sample which had not been extracted with hexane. This value was divided by the time of incubation in minutes and the amount (milligrams) of microsomal protein present in the assay to express the results as the amount (nanomoles) of B[a]P metabolite formed per minute per milligram of microsomal protein.

Protein determination. The protein content of the microsomal preparations was determined by the method of Lowry *et al.* (12). Human serum protein standard (Sigma Chemical Company) was used as the standard reference.

Calculation of the apparent enzyme kinetic parameters, V_{max} and K_m. Enzymatic reaction rates were determined at 8–10 B[a]P concentrations for each microsomal preparation, and the apparent enzyme kinetic parameters, K_m and V_{max}, were calculated using a computer program based on the weighting procedure of Wilkinson (13).

Estimation of f_B. Direct determination of the fraction of the total B[a]P free in the incubation mixture or perfusion medium was not possible using conventional equilibrium dialysis or column chromatography methods. As an alternative, the AHH activity of microsomal preparations of lung and liver from control and 3MC-pretreated rats was determined at two concentrations of added BSA. The concentrations chosen were 1 mg/ml and 32 mg/ml, reflecting the added BSA concentration in the microsomal assay mixtures used in the kinetics experiments and that in the perfusion medium of the isolated organs, respectively. The total B[a]P concentration in these incubations was 0.2 μM, the initial concentration at the beginning of the organ perfusions; f_B was estimated as follows as a correction for effects of binding on clearance:

$$f_B = \frac{\text{AHH activity at 32 mg BSA/ml}}{\text{AHH activity at 1 mg BSA/ml}} \qquad (4)$$

Isolated, perfused organs. The surgical procedures for the removal and perfusion of isolated rat lungs and livers have been described

[2] The abbreviations used are: B[a]P, benzo[a]pyrene; AHH, aryl hydrocarbon hydroxylase; 3MC, 3-methylcholanthrene; BSA, bovine serum albumin.

previously (5, 14). Isolated lungs and livers from both control and 3MC-pretreated rats were perfused at constant flow equivalent to normal organ flow (45 ml/min for lungs; 10 ml/min for livers) with recirculating medium. The 100 ml of medium, prepared just prior to use, consisted of 80 ml of Kreb's bicarbonate buffer containing 4% bovine serum albumin (Pentex, Fraction V, Miles Laboratories, Inc., Elkhart, Ind.) and 20 ml of washed human red blood cells (American Red Cross, Lansing, Mich.) to give a final hematocrit of 20%.

Following an equilibration period during which the inflow perfusion pressure was allowed to stabilize, 20 nmoles of [^{3}H]B[a]P were added as a bolus to the perfusion medium reservoir. Periodically thereafter samples of perfusion medium were removed and analyzed for B[a]P as described below. Lungs were perfused for 60 min following administration of the B[a]P; livers were perfused for 45 min. Following perfusion the organs were removed from the apparatus, blotted, separated from extraneous tissue, and weighed.

Analysis of samples for B[a]P content. B[a]P in the 1.0-ml samples of perfusion medium from isolated lungs was separated from B[a]P metabolites after methanol extraction by high-pressure liquid chromatography according to the method of Selkirk *et al.* (11). The column effluent corresponding to the elution of B[a]P was collected and the radioactivity was quantified by liquid scintillation spectrometry.

Samples (1.0 ml) from liver perfusions were added to 2 ml of 0.15 N KOH in 85% dimethyl sulfoxide and extracted with hexane as described by Van Cantfort *et al.* (10) to separate B[a]P from its metabolites. The extracts containing the [^{3}H]B[a]P were placed in vials, the hexane was evaporated, 10 ml of ACS were added (Amersham, Inc.), and the radioactivity was determined by liquid scintillation spectrometry. Samples analyzed by both methods gave the same results.

Calculation of B[a]P clearance by isolated organs. From semilogarithmic B[a]P disappearance curves for each perfusion experiment, the clearance of B[a]P (Cl_T) was calculated from the following equation:

$$Cl_T = \frac{\text{dose}}{C_0} \times k_e \qquad (5)$$

where k_e is the slope of a plot of the natural logarithm of the reservoir B[a]P concentration versus time determined by linear regression analysis, and C_0 is the initial B[a]P concentration at zero time determined by extrapolation of the linear regression line.

An apparent small clearance of B[a]P occurred during "perfusions" in which no organs were present in the apparatus. This clearance was low as compared with that by the organs with the exception of the clearance by lungs from control rats. The apparent cause was uptake by the tubing. Values for such clearance were determined in a manner identical with that employed when organs were present. For livers this value (mean of two determinations) was 0.48 ml/min, whereas for lungs a value of 1.1 ml/min was obtained. Since Cl_T represents total clearance by the apparatus and the perfused organ, a correction was made for apparatus clearance (Cl_A) to obtain clearance by the perfused organ. Assuming sequential clearance by apparatus and organ, the equation used for this correction was

$$Cl = \frac{Q(Cl_T - Cl_A)}{(Q - Cl_A)} \qquad (6)$$

Concentration dependence of B[a]P elimination. Isolated livers and lungs from 3MC-pretreated rats were perfused at a rate of 10 ml/min as described above except that either 0.56 or 20 nmoles of [^{3}H]B[a]P were added to the perfusion medium reservoir. The amount of radioactivity added was the same at both concentrations.

Distribution of ^{3}H within perfused livers. Isolated livers from control rats were perfused as described above. Twenty nanomoles of [^{3}H]B[a]P were added to the reservoir at zero time. At 15 min after the addition of the B[a]P to the reservoir, the perfusion was ended, the liver was removed from the apparatus, and samples were taken from 23 selected sites. The samples were digested with Soluene-350 (Packard Instrument Company, Inc., Downers Grove, Ill.) and decolorized with 30% hydrogen peroxide; 10 ml of 3a20 scintillation cocktail (Research Products International Corporation, Elk Grove Village, Ill.) were added, and the radioactivity was determined by liquid scintillation spectrometry. Counting efficiency was determined by external standardization.

Determination of portal-hepatic venous shunts. Isolated livers of 3MC-pretreated rats were perfused as described. Following recirculating perfusion with B[a]P, the system was switched to a single-pass mode, and approximately 4.4 μCi of ^{85}Sr-labeled microspheres (20,000 spheres, 15-μm diameter; 3M Company, Medical Products Division, St. Paul., Minn.) were injected into the perfusion tubing in a retrograde manner just upstream from the inflow cannula. The effluent was collected for 4 min and the perfusion was stopped. All tubing and cannulas, as well as the entire liver and effluent perfusion medium, were placed in appropriate vessels and the radioactivity was quantified in a gamma scintillation counter. The counts per minute were summed after subtraction of appropriate blank values, and the fraction of the counts per minute appearing in the effluent was calculated. This value was taken as the fraction of the portal flow which entered the hepatic vein without passing through the hepatic sinusoids.

Statistical analysis. Results are expressed as means ± standard error of the mean and were analyzed by Student's t-test. The level of significance chosen was $p \leq 0.05$ (15).

RESULTS

In initial experiments using liver and lung from control and 3MC-pretreated rats, the time and protein dependence of AHH activity was determined (data not shown). In Table 1 are presented the conditions under which the substrate concentration dependence of the microsomal AHH activity was studied. At the extremes of the B[a]P concentration ranges indicated, product formation was linear with both incubation time and microsomal protein concentration. The incubation times and microsomal protein concentrations chosen for the substrate concentration-dependence studies were within these linear time- and protein-dependence regions. Under the conditions indicated, 10% or less of the substrate was usually metabolized during the incubations. In no case was more than 20% of the B[a]P metabolized. Thus, these rates reflect initial reaction rates for the specified conditions. The B[a]P concentration ranges for determination of sub-

TABLE 1

Conditions under which the apparent enzyme kinetic constants for the microsomal metabolism of B[a]P were determined

In initial experiments at the extremes of the B[a]P concentration ranges, linear dependence of B[a]P metabolism on incubation time and microsomal protein concentration were observed. The times and protein concentrations used in the concentration-dependence assays were within these linear regions.

	Control		3MC pretreatment	
	Lung	Liver	Lung	Liver
Incubation time (min)	4	4	2	2
Microsomal protein (μg/ml)	614 ± 17	20 ± 1	209 ± 18	0.98 ± 0.05
B[a]P concentration range (μM)	0.1–2.0	2.0–20.0	0.1–2.0	0.04–0.55

strate dependence were selected to be approximately 0.2 and 2.0 times the approximate apparent K_m value determined in preliminary experiments.

In Fig. 1 are presented the results of the B[a]P concentration-dependence assays for the microsomal metabolism of B[a]P by liver and lung from control and 3MC-pretreated rats. These Lineweaver-Burk plots appeared linear.

Using the activity and substrate concentration data from such microsomal incubations the apparent enzyme kinetic constants, K_m and V_{max}, were calculated (Table 2). The microsomal preparation of control lung had a lower apparent K_m than did the microsomal preparation of control liver. However, when organs from 3MC-pretreated rats were used, the situation was reversed; liver had a K_m 4 times less than that of the lung microsomes. Pretreatment of the rats with 3MC did not alter the apparent K_m of lung microsomes.

Per milligram of microsomal protein, liver microsomes had a much greater activity than those of lung in both control and 3MC-pretreated rats, being about 140 and 60 times greater, respectively. When the data were expressed in terms of the metabolic activity of the whole organ, liver was by far the organ of greater metabolic activity, having V_{max} values 2500 and 1200 times greater than lung in control and 3MC-pretreated rats, respectively. However, 3MC pretreatment had a greater effect on lung than on liver, stimulating this organ's metabolic activity 8 times while raising liver activity only half as much.

Under first-order conditions, Cl'_{int} is probably a better index of the metabolic capacity of the organ than the V_{max} alone, since it also takes into account the affinity of the enzyme for its substrate. Cl'_{int} for lungs of control rats was 0.9 ml/min, which increased to 6 ml/min in rats pretreated with 3MC. Similarly, livers of control rats had a Cl'_{int} of 73 ml/min, whereas that of 3MC-pretreated rats was 28 liters/min. Accordingly, it appears that in both control and 3MC-pretreated rats liver has a much greater Cl'_{int} than lung.

According to the perfusion-limited model of clearance, several factors limit the ability of organs to obtain these maximal clearances. These factors include the blood flow to the organ and the free fraction of the compound in the blood.

Pulmonary blood flow is considerably greater than hepatic flow, since lungs receive the entire cardiac output whereas liver has a flow only 25% as large. For the size of rats used in these studies we have estimated normal pulmonary blood flow to be 45 ml/min and normal hepatic flow to be 10 ml/min (14, 16). 3MC pretreatment does not affect total hepatic flow (3).

Binding of substrates to plasma proteins and other blood components reduces the free fraction of these compounds in blood, and thus may decrease the amount available for metabolism. We attempted unsuccessfully

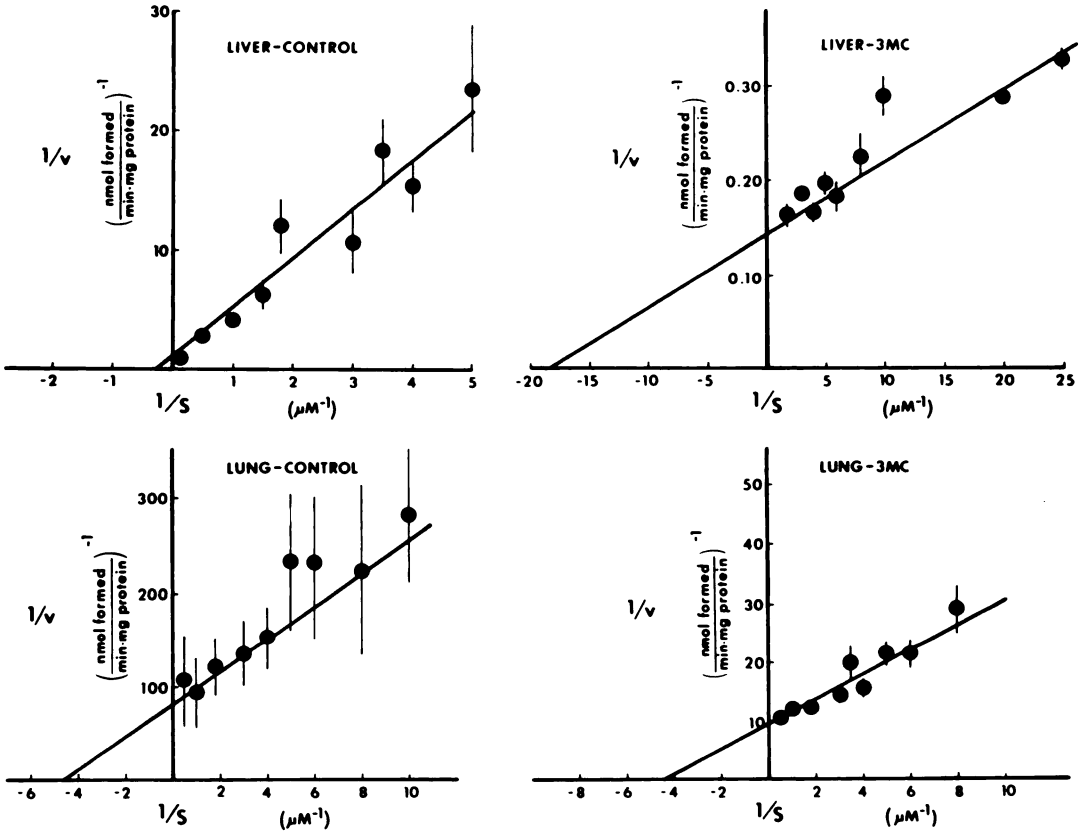

FIG. 1. *Lineweaver-Burk plots of the B[a]P concentration dependence of the velocity of reaction for microsomal AHH activity in liver and lung from control and 3MC-pretreated rats*

Details of the treatment procedures, incubations, and assay techniques are described under Methods. The data points represent the mean ± standard error of the mean of three experiments. S, substrate concentration; v, velocity of reaction.

TABLE 2

Apparent enzyme kinetic parameters and intrinsic free clearance for microsomal B[a]P metabolism

Metabolic activity of microsomes from lungs and livers of three corn oil-pretreated and three 3MC-pretreated rats was assessed by the method of Van Cantfort *et al.* (10) and analyzed by the procedure of Wilkinson (13).

	Control		3MC pretreatment	
	Lung	Liver	Lung	Liver
K_m (μM)	0.22 ± 0.08	5.5 ± 1.1	0.23 ± 0.03	0.054 ± 0.007
V_{max} [a]	0.012 ± 0.003	1.7 ± 0.5	0.107 ± 0.003	6.8 ± 0.4
V_{max} [b]	0.16 ± 0.05	409 ± 100	1.3 ± 0.1	1524 ± 256
Cl'_{int} [c] (ml/min)	0.9 ± 0.4	73 ± 3	6.0 ± 1.2	28,257 ± 3006

[a] Nanomoles of B[a]P metabolites formed per minute per milligram of microsomal protein.
[b] Nanomoles of B[a]P metabolites formed per minute per organ.
[c] $Cl'_{int} = V_{max}/K_m$

to measure the plasma protein binding of B[a]P by two conventional methods, equilibrium dialysis and column chromatography. In the former method, equilibrium could not be reached even after 6 days, whereas in the latter method the B[a]P bound avidly to the column material. Therefore, we opted to assess the influence of this parameter on Cl'_{int} by determining the effect of added BSA on the microsomal metabolism of B[a]P. This is shown in Fig. 2 for the metabolism of B[a]P by microsomes of livers from control rats. No change in AHH activity was observed with increasing concentrations of BSA up to 1 mg/ml. At this concentration AHH activity began to decrease with increasing BSA concentration.

This effect was also examined at two BSA concentrations for each of the organs and pretreatments (Table 3). The concentrations of added BSA were those present in the enzyme kinetic assays (1 mg/ml) and used in the isolated organ perfusion medium (32 mg/ml). The B[a]P concentration used was the nominal initial concentration in the perfusions, 0.2 μM. AHH activity in microsomes of livers from control rats was significantly reduced by increasing the BSA concentration while activity in liver microsomes from 3MC-pretreated rats was somewhat increased. Activity in lung microsomes from both control and 3MC-pretreated rats was not changed.

Using the weights of the organs used in the perfusion studies, Cl'_{int} of these organs was calculated from the enzyme kinetic parameters of Table 2 according to Eq. 1. These values of Cl'_{int} and normal organ flow were inserted into the clearance equation (Eq. 2) to predict the clearance of B[a]P by liver and lung. Since binding to BSA could not be directly determined, its influence on maximal clearance was taken into account by substituting for f_B the ratio of the microsomal AHH activities at 32 and 1 mg of added BSA per milliliter of incubation medium (Table 3 and Methods). The resultant predictions of clearance are presented in Table 4 along with the clearances actually observed in the isolated organs perfused at normal organ flows.

The clearance values determined in the isolated lungs were not significantly different from those predicted by

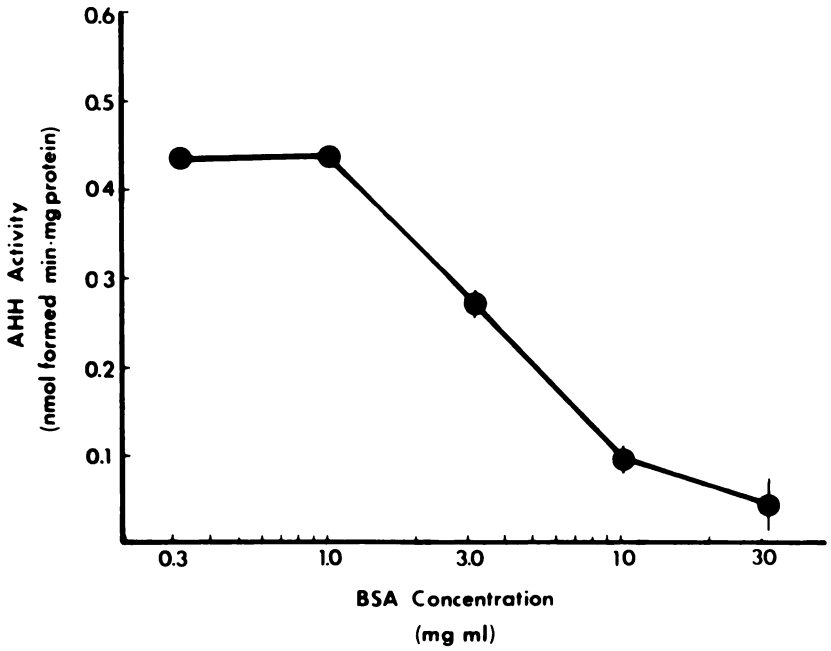

FIG. 2. *Influence of added BSA on the AHH activity of liver microsomes from control rats*

Microsomes from livers of control rats were incubated as described under Methods with increasing concentrations of BSA, and the AHH activity was determined. The B[a]P concentration was 1.0 μM. Results are mean activity ± standard error of the mean of three experiments.

TABLE 3

Effect of added BSA on the microsomal metabolism of B[a]P

AHH activity of microsomal preparations determined according to the method of Van Cantfort *et al.* (10). Results represent mean ± standard error of the mean of three experiments. The B[a]P concentration was 0.2 μM.

Tissue and treatment	AHH activity[a]		Ratio[b]
	Added BSA 1 mg/ml	Added BSA 32 mg/ml	
Liver			
Corn oil	53 ± 5	7.9 ± 2.4[c]	0.14 ± 0.03
3MC	3,660 ± 50	5,210 ± 290[c]	1.4 ± 0.1
Lung			
Corn oil	3.1 ± 0.8	3.5 ± 1.1	1.1 ± 0.1
3MC	44 ± 1	45 ± 2	1.0 ± 0.0

[a] Nanomoles of B[a]P metabolites formed per minute per milligram of microsomal protein.

[b] Ratio of activity at 32 mg/ml added BSA to that at 1 mg/ml added BSA.

[c] Significantly different from activity at 1 mg/ml added BSA by Student's *t*-test ($p < 0.05$).

the model. For livers, a small but statistically significant difference between the observed and predicted means was obtained. B[a]P clearance by lung of control rats at normal pulmonary flow as low but was increased by 3MC pretreatment. Hepatic clearance was high for livers perfused at normal flow from both control and 3MC-pretreated rats. Thus, in control rats hepatic clearance was much greater than pulmonary clearance, whereas in 3MC-pretreated rats lung and liver clearances were about equal.

TABLE 4

Clearance of B[a]P at normal organ flow as predicted from microsomal metabolic activity and as observed in isolated perfused organs

	Clearance			
	Control		3MC pretreatment	
	Lung	Liver	Lung	Liver
	ml/min			
Predicted[a]	0.97 ± 0.14	4.6 ± 0.5	7.0 ± 0.8	10.0 ± 0.1
Observed[b]	0.99 ± 0.10	5.9 ± 0.2[c]	8.9 ± 0.5	6.7 ± 0.6[c]

[a] Clearance predicted according to the perfusion-limited model (Eqs. 1 and 2) using values of Cl'_{int} from Table 2, the estimates of f_B from Table 3, and normal organ flows of 10 ml/min for liver and 45 ml/min for lung (14, 16). Values are mean clearance values ± "SEM" based on the sum of the variances of the individual variables relative to their means, i.e.:

$$\frac{S^2_{Cl}}{\bar{X}_{Cl}} = \frac{S^2_{Cl'_-}}{\bar{X}_{Cl'_-}} + \frac{S^2_{f_B}}{\bar{X}_{f_B}} + \frac{S^2_{\text{organ wt}}}{\bar{X}_{\text{organ wt}}}$$

$$\text{"S.E.M."} = (S^2_{Cl}/N)^{1/2}$$

where S^2 represents variance, $\bar{X}$ the mean, and N the number of values used in the composite variance determination. Results are from four to six isolated organs per group.

[b] Clearance in isolated organs calculated as the product of the first-order disappearance rate constant and the apparent volume of distribution when perfused at normal organ flows (10 ml/min for liver; 45 ml/min for lung).

[c] Significantly different ($p < 0.05$) from predicted clearance (Student's *t*-test, paired design).

Because hepatic Cl'_{int} is so large, the perfusion-limited model predicts that hepatic B[a]P clearance should be equal to the organ flow in 3MC-pretreated rats (Table 4). However, B[a]P clearance by the isolated liver was somewhat less than the flow. We investigated several possible explanations for this difference:

1. One possibility was that B[a]P clearance was concentration-dependent. Therefore, B[a]P clearance was measured in isolated organs of 3MC-pretreated rats perfused at initial B[a]P concentrations of 200 or 5.6 nM. This lower concentration is 10 times less than the K_m value for liver microsomes of 3MC-pretreated rats (Table 2). As seen in Table 5, no difference was observed between the B[a]P clearance values obtained at the two initial concentrations in either organ.

2. Another possibility was that liver was perfused in a nonhomogeneous manner. This was assessed by determining the concentration of ^3H in various sites throughout the liver following 15 min of perfusion with [^3H]B[a]P. No significant differences were observed in the concentration of the label among 23 sites in the perfused liver.

3. A third explanation of why hepatic clearance was less than predicted was that possibly a shunt was directing a portion of the flow from the portal vein into the venous outflow of the liver and bypassing the sinusoids. When 15-μm diameter microspheres were injected into the inflow of isolated livers, only about 7 of 20,000 microspheres (0.035%, ±0.021, $N = 4$) appeared in the outflow. Therefore, shunting of flow around the liver sinusoids was unlikely.

DISCUSSION

The results of this study demonstrate that quantitative knowledge of the factors involved in the perfusion-limited model of clearance (i.e., flow, free fraction in the blood, and intrinsic free clearance) permit a useful prediction of the ability of liver and lung to clear circulating B[a]P. Good agreement was found between the clearances predicted by the model and those observed in the isolated organs perfused at normal organ flows (see Table 4). This study, then, adds to the evidence that enzyme kinetic data can be used to predict the ability of the whole organ to remove circulating drugs and other xenobiotic compounds (1, 5–7).

Apparent enzyme kinetic parameters, V_{max} and K_m, for the microsomal metabolism of B[a]P by liver (17–21) and lung (22) of control and 3MC-pretreated rats have been reported previously. The K_m values determined in these studies are for the most part considerably greater than those which we obtained in our experiments. For example, Zampaglione and Mannering (19) reported K_m values for the hepatic microsomal metabolism of B[a]P of 14.6 and 2.9 μM for control and 3MC-pretreated rats, respectively. In contrast, the values we determined were 5.5 and 0.05 μM for these two conditions. These differences could be due to a number of causes, such as the assay method used or the conditions under which the activity was measured. In all but one of the studies cited above, the assay for the production of B[a]P metabolites involved detection of fluorescent metabolites. Even if care were taken to differentiate the fluorescence of

TABLE 5

Concentration dependence of B[a]P clearance by isolated lungs and livers of rats pretreated with 3MC

Lungs and livers were perfused at constant flow (10 ml/min) with recirculating medium as described under Methods.

	Initial B[a]P concentration	k_e[a]	V_d[b]	Clearance[c]	N
	nM	*min*$^{-1}$	*ml*	*ml/min*	
Lung	200	0.051 ± 0.002	102 ± 11	4.6 ± 0.4	6
	5.6	0.053 ± 0.004	110 ± 22	5.4 ± 1.1	3
Liver	200	0.072 ± 0.006	96.1 ± 3.5	6.4 ± 0.6	5
	5.6	0.083 ± 0.007	81.4 ± 8.5	6.3 ± 1.1	3

[a] First-order rate constant of elimination = slope of logarithm of concentration versus time curve.

[b] Apparent volume of distribution calculated as the dose divided by the concentration at zero time determined by extrapolation of the disappearance curve.

[c] Clearance = $k_e \times V_d$. Clearance corrected for "no organ clearance."

B[a]P from that of hydroxylated metabolite, such as in the study of Robie *et al.* (21), the amount of product produced could be underestimated if (a) some products produce less fluorescence than others, (b) subsequent metabolism removes the ability to fluoresce, or (c) some metabolites are not fluorescent. This is especially true when comparing metabolite production among organs or between species, where the cytochrome P-450 isozyme populations may differ markedly. Metabolite production could also be overestimated if the major metabolite generates a greater intensity than the standard used to quantify the results. In contrast, the radiometric assay used in this investigation measures all of the metabolites by quantitative and selective removal of the unreacted substrate from the incubation medium. Thus, no matter which metabolite(s) is produced, it is quantified even if it undergoes further metabolism.

Another difference from previous studies was the amount of microsomal protein present in the assay mixture. In contrast to previous studies, in which in most cases liver microsomal protein was of an assay concentration of 0.5–2.0 mg/ml, we used at least 25 times less (see Table 1). In this way the substrate-to-enzyme concentration ratio is more favorable for the determination of initial rates. Additionally, differences from previously reported apparent K_m values may be due to a difference in the B[a]P concentration range used in our experiments.

It is obvious from the maximal velocities (Table 2) that liver in both control and 3MC-pretreated rats has the greater enzymatic activity. This is true for both specific (per milligram of microsomal protein) activity as well as whole organ activity. It is difficult to compare the V_{max} values we obtained with those reported in the literature since the values for liver microsomal AHH activity range from 50 to 3000 and from 500 to 8000 pmoles of products formed per minute per milligram of microsomal protein for control and 3MC-pretreated rats, respectively (8, 19–21, 23). Our results agree better with those in which high-pressure liquid chromatographic and radiometric assay methods were used than with those in which fluorometric assays were performed. Similarly, our V_{max} values obtained from lung microsomes are close to those of Prough *et al.* (23) but are less than those obtained by Vadi *et al.* (22).

Investigations using various inducers of microsomal enzyme activities have established that cytochrome P-450 exists in several isozymic forms in both liver and lung (24, 25). This differential induction is reflected in altered enzyme activities toward some, but not all, substrates (26, 27), suggesting that either the relative amount of a particular isozyme has been increased or that a new isozyme is being produced. The results from this study suggest that 3MC may not produce the same effect in both lung and liver. In liver, 3MC reduced the apparent K_m of AHH for B[a]P. However, in lung this parameter remained unchanged. This suggests that in liver a new isozyme may have been produced, while in lung the amount of a constitutive form was increased. Alternatively, the form from induced liver may have been present in control microsomes, but was insignificant relative to the others, so that its low K_m was not observed. It is not possible from our data to distinguish between these possibilities. However, the results of Pickett *et al.* (28) indicating that a new messenger RNA, not detectable in livers from control rats, is produced following 3MC pretreatment suggest that the former explanation is probable.

The intrinsic free clearance term depends upon the qualities of the degradative enzyme. The V_{max} portion of the term reflects the amount of enzyme present in the organ whereas the apparent K_m reflects the affinity of the enzyme for the B[a]P. Both of these qualities are important for removal of the B[a]P from the circulating blood under first-order conditions. Thus, the intrinsic free clearance provides a better over-all estimate of the metabolic capacity of the intact organ than does enzyme activity (i.e., V_{max}) alone. This is especially true when changes occur in both components such as occurred with 3MC induction in rat liver.

Direct determination of the free fraction of B[a]P in either the microsomal incubations or the perfusion medium was not possible. To estimate the effect that BSA protein concentration might have on the ability of the microsomal enzymes to metabolize B[a]P and therefore of the isolated organs to clear circulating B[a]P, the relative enzymatic activities of the microsomal preparations were compared at two concentrations of added BSA (Table 3). In the microsomal preparation with the highest K_m (control liver), increasing the concentration of BSA reduced AHH activity seven-fold (Fig. 2; Table 3). In the other microsomal preparations, each of which had a relatively lower apparent K_m than control liver microsomes, the rate of B[a]P metabolism was not reduced by

increasing the BSA concentration. One explanation for these results is that the affinity of B[a]P for BSA is such that a change in BSA concentration from 1 to 32 mg/ml had a marked effect on AHH activity when affinity of AHH for B[a]P was low but not under conditions where AHH affinity for B[a]P was high. The microsomal preparation with the very lowest K_m came from livers of 3MC-pretreated rats. Increasing the BSA protein concentration actually increased slightly the metabolism rate by these microsomes. The reason for this cannot be determined from our data, but it could be that binding of B[a]P to BSA may have in some manner facilitated delivery of this substrate to the enzyme.

We did not examine the effects of red blood cell binding by B[a]P in this study. Our unpublished studies indicate that changing the hematocrit from 20% to 36% does not affect B[a]P clearance by isolated lungs. Elimination of BSA from the perfusion medium, however, has a large effect on clearance.

The results of the perfused organ experiments suggest that enzyme activity or metabolic capacity determined from microsomal studies alone is not sufficient to predict directly an organ's contribution *in vivo* to the removal of metabolically eliminated compounds from the circulation. That is, prediction from V_{max} or Cl'_{int} values alone (Table 2) would lead to the conclusion that in both control and 3MC-pretreated rats liver would predominate in the elimination of B[a]P from the body *in vivo*. However, when the constraints of organ blood flow and protein binding are accounted for, as in the perfusion-limited model, a different prediction emerges. In control rats even though the metabolic capacity (Cl'_{int}) is 80 times greater, liver clearance is predicted to be only about 5 times greater than that of lung (Table 4). In 3MC-pretreated rats, where the Cl'_{int} of liver is nearly 5000 times greater than that of lung, clearance of B[a]P by the two organs is about equal.

These predictions are supported by experiments in isolated organs. B[a]P clearance by perfused lungs was accurately predicted by our studies using microsomes. Small differences were obtained between predicted and observed values in perfused livers, the observed values for livers of 3MC-pretreated rats being somewhat lower than the prediction, whereas that for control rats was somewhat greater. We examined several possible explanations for the small discrepancy in the isolated livers of 3MC-pretreated rats: (a) that clearance may be concentration-dependent in livers with AHH of low K_m, (b) that distribution of the B[a]P within the perfused liver may be nonuniform, or (c) that shunting of the perfusion medium may occur around the hepatic sinusoids directly into the venous outflow of the liver.

One of the primary requirements of the perfusion-limited model is that the free concentration of the cleared compound be less than the K_m value of the degradative enzyme (4). In other words, that clearance occurs under first-order conditions. The initial B[a]P concentration in the perfusion medium of the isolated organs (0.2 μM) was equal to, or greater than, the apparent K_m values of lung and liver microsomes from 3MC-pretreated rats. The fact that the disappearance curves were linear suggested that elimination was first-order. However, to test further whether this was true, we perfused isolated lungs and livers at a concentration well below the K_m value (Table 5). There was no difference between the clearance obtained at the higher concentration and that observed at the lower concentration.

A second possibility was that nonuniform distribution of the flow, and thus delivery of B[a]P, occurred in the isolated livers. For example, it might have been that certain areas of the liver were not perfused. This would reduce the effective mass of the liver and concomitantly the effective Cl'_{int}. However, no significant differences in the concentration of tritium were found among 23 selected sites throughout the liver, suggesting that delivery of B[a]P was uniform.

A third possibility was that a significant fraction of the flow to the isolated liver was shunted around the hepatic sinusoids from the portal supply directly into the hepatic veins. To examine this, radioactively labeled microspheres were injected into the inflow perfusion medium. They were injected against the direction of flow in order to assure adequate mixing. Microspheres of the size used in this experiment (15 μm diameter) are trapped within the liver if they enter the sinusoids. If flow is shunted, some microspheres should appear in the effluent perfusion medium. Since less than 0.1% of the radioactivity appeared in the effluent medium, these results suggest that there was minimal anatomical shunting of portal flow to the hepatic veins within the perfused rat liver.

Other possibilities exist for the small discrepancies observed between predicted and experimentally determined clearance values. For example, in our predictions we did not correct estimations of intrinsic clearance for microsomal yield. Microsomal yield would affect the intrinsic clearance value proportionately. Low microsomal yield would introduce the most error into the predicted clearance value for control lung, since its clearance is most dependent upon intrinsic clearance. In the other three conditions, clearance was more dependent upon flow and influenced little by moderate increases in intrinsic clearance. The fact that the predicted values were so close to those which were determined experimentally suggests that microsomal yield was reasonably high. Other possibilities for differences between predicted and observed values include cofactor limitations of metabolic activity, inactive areas within the liver, underestimation of the contribution of B[a]P binding to blood elements, or the rate of dissociation from blood elements limiting the diffusion of B[a]P into the organ. In addition, the interaction of albumin-bound B[a]P at hepatocyte albumin receptors may be important for delivery of B[a]P into liver cells, as has been demonstrated for taurocholate (29). Perhaps the concentration of albumin used competitively inhibits B[a]P delivery. We have not as yet examined these possibilities. However, it should be remembered (1) that an exact quantitative prediction may not be possible. Estimation of metabolism in an intact organ from enzyme activities determined under optimal time, temperature, pH, and cofactor concentrations in broken-cell preparations is a large extrapolation. Despite the large number of factors to account for, the results of our study suggest that such extrapolations may rather accurately predict the participation of organs in the metabolic elimination of circulating xenobiotic compounds.

Our results suggest that both lung and liver may have

important roles in the metabolic disposition of B[a]P. The exact roles of these organs will depend upon the route of exposure. For example, following an oral dose, liver will be responsible for metabolizing nearly all the administered B[a]P since hepatic first-pass extraction is high. However, B[a]P elimination after exposure by other routes not influenced by hepatic first-pass are subjected to pulmonary first-pass effects. In this case, the contribution of the lungs to total body elimination is governed by their intrinsic enzyme activity (i.e., pulmonary intrinsic clearance) whereas the contribution of the liver will be determined by hepatic clearance (30), which in the case of B[a]P is determined largely by blood flow. In the 3MC-pretreated animal this may result in a nearly equal contributiuon of these two organs to total body B[a]P clearance.

In summary, we have investigated the usefulness of enzyme kinetic data and the perfusion-limited model of clearance to predict the ability of isolated organs of rats to clear circulating B[a]P. The prediction was based not only on apparent enzyme kinetic parameters assessed in broken-cell preparations, but also accounted for influences of organ perfusion and for binding of B[a]P to protein in the perfusion medium. The prediction was tested in isolated rat lung and liver preparations perfused at normal organ flows. We conclude that, in both control and 3MC-pretreated rats, liver has the greater metabolic capacity when assessed in microsomal preparations. However, in the intact organ, where influences of flow and protein binding are important, this disparity in metabolic capacity is not always reflected in differences in organ clearance. In fact, lung from 3MC-pretreated rats is about equal to liver in its ability to clear circulating B[a]P. The results of this study indicate that lung may play a greater role in the metabolism of circulating xenobiotic compounds than is suggested by its metabolic capacity alone.

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