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# BENZO(*a*)PYRENE HYDROXYLASE FROM *SACCHAROMYCES CEREVISIAE*

## SUBSTRATE BINDING, SPECTRAL AND KINETIC DATA

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## Summary

*Saccharomyces cerevisiae*, brewer's yeast, produces a microsomal benzo(*a*)-pyrene hydroxylase when grown at high glucose concentrations of which the haemoprotein, cytochrome *P*-450 (RH, reduced-flavoprotein:oxygen oxido-reductase (RH-hydroxylating) EC 1.14.14.1) is a component. We report here kinetic data derived from Lineweaver-Burk plots of benzo(*a*)pyrene hydroxyla-tion. The Michaelis constant was decreased by growth of the yeast in the pres-ence of benzo(*a*)pyrene showing the induction of a form of the enzyme more specific for this compound. NADPH or cumene hydroperoxide could be used as cofactors by this enzyme, although with different $K_m$ and $V$ values for benzo(*a*)pyrene. A solubilised and a solubilised, immobilised enzyme prepara-tion were capable of benzo(*a*)pyrene hydroxylation, using cumene hydro-peroxide but not NADPH as the cofactor.

Benzo(*a*)pyrene was found to produce a modified type I spectral change with yeast and rat liver microsomes. The interaction of benzo(*a*)pyrene with cytochrome *P*-450 was investigated further by means of an equilibrium gel filtration technique. There appeared to be 20 binding sites per mol of cyto-chrome *P*-450 for benzo(*a*)pyrene, in both yeast and rat liver microsomes.

## Introduction

We have described the production of cytochrome *P*-450 (RH, reduced-flavo-protein:oxygen oxidoreductase (RH-hydroxylating) EC 1.14.14.1) by *Sac-*

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazolyl-2-)-benzene.

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*charomyces cerevisiae* [1] and have also identified the benzo(*a*)pyrene metabolites formed by the action of this enzyme, which were 7,8-dihydro-7,8-dihydroxybenzo(*a*)pyrene, 9-hydroxybenzo(*a*)pyrene and 3-hydroxybenzo(*a*)-pyrene [2].

The major pathways for the metabolism of benzo(*a*)pyrene and other polycyclic aromatic hydrocarbons in mammalian tissues involve the aryl hydrocarbon hydroxylase system (RH, reduced-flavoprotein:oxidoreductase (RH-hydroxylating) EC 1.14.14.1), epoxide hydratase and glutathione *S*-epoxide transferase (EC 2.5.1.18) [3,4]. Aryl hydrocarbon hydroxylase is the first of these enzymes and introduces an oxygen atom into its substrates to produce epoxides [4—9]. It has been known for some time that aryl hydrocarbon hydroxylase and microsomal mixed-function oxidase are the same [3,10]. The kinetics of mammalian aryl hydrocarbon hydroxylase have been investigated by a number of workers [11—17] and changes in the kinetics after pretreatment of the animal with polycyclic aromatic hydrocarbons have been demonstrated [12—17].

The technique of difference spectrophotometry has been used widely for more than 10 years to study the spectral changes which result from the binding of drugs and other compounds to liver microsomal cytochrome *P*-450 [18—20].

The interactions of drugs and proteins have been studied by a number of different equilibrium methods but the one described here, that of equilibrium gel filtration, has not been widely used [21—23].

## Materials and Methods

### *Measurement of benzo(a)pyrene hydroxylase*

*Saccharomyces cerevisiae* (N.C.Y.C. No. 240) was grown in 20% glucose medium and benzo(*a*)pyrene hydroxylase activity was measured as previously described [2].

### *Solubilisation and immobilisation of yeast cytochrome P-450*

The method used to solubilise yeast cytochrome *P*-450 was modified from that of Yoshida et al. [24]. Sodium cholate, EDTA and dithiothreitol were dissolved in 5000 × *g* supernatant derived from disrupted yeast, to give final concentrations of 10 g/l, 0.5 g/l and 0.3 g/l (w/v), respectively. The solution was mixed on a roller-mixer in a stoppered test-tube at 4°C for 1 h and then centrifuged at 160 900 × *g* in a Beckman L5-65 Ultracentrifuge. In a typical experiment approximately 50% of the cytochrome *P*-450 was recovered, all of it being in the supernatant, using 5 g of yeast (12 nmol cytochrome *P*-450) the 5000 × *g* fraction contained 0.6 nmol enzyme/ml (volume 10 ml).

Cytochrome *P*-450 was immobilised onto microcrystalline cellulose as has been shown previously (Gondal, J.A., unpublished data). 10 ml of 5000 × *g* supernatant was solubilised as described but not centrifuged. 1.5 g of cellulose was added to the solution and then mixed for 1 h on a roller-mixer. Cross-linking was accomplished by adding 0.3 ml of 0.25 M glutaraldehyde solution (final concentration was 7.5 mM) and mixing for a further hour. After this time the cellulose was separated by centrifugation in a MSE High-Speed 18 centri-

fuge at 12 000 × *g* (10 000 rev./min) for 10 min. From 1.5 g of dry cellulose approx. 6 g of undried immobilised enzyme was produced. There was 0.45 nmol cytochrome *P*-450/g cellulose (74% recovery).

*Spectral binding of benzo(a)pyrene to cytochrome P-450*

The spectral changes resulting from the addition of benzo(*a*)pyrene to microsomal suspensions were recorded using a Perkin-Elmer 356 Two-Wavelength Double Beam Spectrophotometer (Split-Beam Mode). A double-cell technique similar to that described by Goujon et al. [25] was employed in order to remove interference from the benzo(*a*)pyrene which absorbs in the wavelength range used (350—500 nm). Spectral titrations at fixed wavelengths were subjected to double-reciprocal plots and the spectral dissociation constant ($K_s$) determined [26]. The absorbance change used was $A_{415nm} + A_{385-375nm}$ due to changes in the baseline during the addition of benzo(*a*)pyrene, and the $K_s$ values were determined by least-squares regression analysis.

*Equilibrium gel filtration of the benzo(a)pyrene-cytochrome P-450 complex*

A column was made from a Pasteur pipette by placing a plug of glass wool in the bottom and packing with Sephadex G-25 (column dimensions were 5 × 45 mm). The column was equilibrated with 0.2 M phosphate buffer, pH 7.0, containing 10% dioxan (w/v) and benzo(*a*)pyrene in a range of concentrations. The benzo(*a*)pyrene in the buffer consisted of a fixed, known amount of [G-$^3$H]-benzo(*a*)pyrene and various known amounts of unlabelled benzo(*a*)pyrene, the specific activity of the benzo(*a*)pyrene in the buffer was determined by counting the radioactivity of a small sample.

Once the column was equilibrated with the benzo(*a*)pyrene-containing buffer, usually after passing 20 to 30 ml, a small volume of microsomal suspension, usually 10 µl, was applied to the top of the column and 2-drop (0.06 ml) samples were collected. These samples were counted in a Packard Tri-Carb Scintillation Spectrometer after adding 4 ml of toluene/metapol (2 : 1) scintillant containing 0.5% PPO and 0.02% dimethyl POPOP (w/v). The binding of the benzo(*a*)pyrene to the cytochrome *P*-450 in the microsomal fraction resulted in an increase in the radioactivity when the protein was eluted. By using the known specific activity of the benzo(*a*)pyrene it was possible to calculate the amount bound. The experiment was repeated at increasing benzo(*a*)pyrene concentrations (decreasing specific activity) and by making a Scatchard plot it was possible to calculate the apparent association constant for each binding site and the number of binding sites per mol of cytochrome *P*-450.

*Materials*

Sodium cholate, EDTA, dithiothreitol, microcrystalline cellulose and benzo(*a*)pyrene were obtained from The Sigma Chemical Company (London) Ltd., Poole, Dorset. Glutaraldehyde (25% for electron microscopy) was supplied by BDH Chemicals Ltd., Poole, Dorset. [G-$^3$H]Benzo(*a*)pyrene was obtained from The Radiochemical Centre, Amersham, Bucks. PPO and dimethyl POPOP were supplied by The Packard Instrument Co. Inc., IL, U.S.A. and metapol was obtained from Durham Chemicals Distributors Ltd., Birtley,

Tyne and Wear. Sephadex G-25 was supplied by Pharmacia (Great Britain) Ltd., Hounslow, Middlesex.

## Results and Discussion

*Yeast benzo(a)pyrene hydroxylase*

*(i) NADPH-supported benzo(a)pyrene hydroxylase.* The enzyme was found to have a broad pH optimum in the region from 6.5 to 7.0, which is lower than that found in mammalian systems. For example, Nebert and Gelboin [11] found the pH optimum in mammalian cell culture to be pH 7.5 and this pH was also used by Robie et al. [16] in experiments using rat liver microsomes. Alvares et al. [27] and Gurtoo et al. [28] used pH 7.4 for their experiments with rat liver microsomes. Cumps et al. [17] showed that the pH optimum for rat liver microsomes was 7.8.

The thermal stability of the yeast benzo(*a*)pyrene hydroxylase was compared with that of cytochrome *P*-450 as measured spectrophotometrically by the carbon monoxide-binding spectrum. (Fig. 1) Plotting $\log_{10}$ of the activities of benzo(*a*)pyrene hydroxylase and cytochrome *P*-450 against time at 50°C gave two identical straight lines. This means that yeast benzo(*a*)pyrene hydroxylase and cytochrome *P*-450 have the same thermal stability and implies that cytochrome *P*-450 is part of the benzo(*a*)pyrene hydroxylase system, as in mammalian systems. The thermal stability of the yeast benzo(*a*)pyrene hydroxylase was very similar to that of the enzyme measured in mammalian cell culture by Nebert and Gelboin [11].

The kinetics of the yeast NADPH-supported benzo(*a*)pyrene hydroxylase were investigated by means of double-reciprocal Lineweaver-Burk plots of the rate of metabolite formation vs. benzo(*a*)pyrene concentrations. Experiments

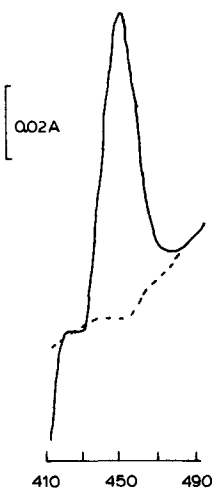


**Fig. 1. The carbon monoxide-binding spectrum of yeast microsomal fraction. ------, Baseline; ———, CO-difference spectrum. The peak is at 449 nm.**

TABLE I

Values of the $K_m$ (mM) and $V$ (pmol 3-hydroxybenzo(*a*)pyrene formed per nmol cytochrome *P*-450 per h) for NADPH- and cumene hydroperoxide-supported yeast benzo(*a*)pyrene hydroxylase.

| Cofactor | NADPH | | | Cumene hydroperoxide | | |
|---|---|---|---|---|---|---|
| Pretreatment | $K_m$ | $V$ | Correlation coefficient | $K_m$ | $V$ | Correlation coefficient |
| Control | 0.45 | 15.1 | 0.988 | 3.00 | 286 | 0.974 |
| Sodium phenobarbitone | 0.53 | 19.7 | 0.999 | 3.00 | 357 | 0.993 |
| Benzo(*a*)pyrene | 0.17 | 26.1 | 0.989 | 0.36 | 63 | 0.999 |

were performed using the enzyme from yeast grown in normal 20% glucose growth medium (control) and also from that grown in medium containing, in addition, either 6 mM sodium phenobarbitone or 20 $\mu$M benzo(*a*)pyrene. Table I gives the values for the Michaelis constant ($K_m$) and maximum rate ($V$) obtained from double-reciprocal plots, by least-squares regression analysis. The correlation coefficient, which is a measure of how well the points fit the regression line, is also given; the best fit possible would have a coefficient of 1.000.

It can be seen that pretreatment of the yeast with benzo(*a*)pyrene caused a decrease in the $K_m$ from 0.45 to 0.17 mM, whereas sodium phenobarbitone pretreatment did not have any significant effect on the $K_m$. In an experiment

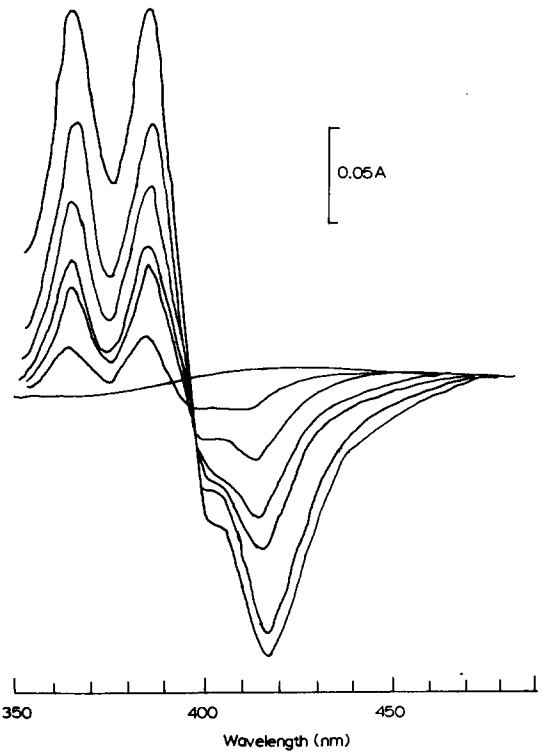


Fig. 2. The binding spectrum of benzo(*a*)pyrene with yeast microsomal fraction.

in which yeast was pretreated with 4 $\mu$M benzo(*a*)pyrene, no difference in the $K_m$ was produced compared with the control. A decrease in the $K_m$ after pretreatment with a polycyclic aromatic hydrocarbon has been described by Alvares et al. [12] and by Kuntzman et al. [13].

Table I also shows that pretreatment of the yeast with benzo(*a*)pyrene caused an increase in the $V$ value, an effect which is in agreement with the observations of many workers, since pretreatment of rats with benzo(*a*)pyrene or 3-methylcholanthrene usually stimulates benzo(*a*)pyrene hydroxylase activity [29—32], the exception being, where no change in $V$ was found after pretreatment of rats with 3-methylcholanthrene [17].

*(ii) Cumene hydroperoxide-supported benzo(a)pyrene hydroxylase.* It was found that cumene hydroperoxide, an oxygen donor, which has been previously used in steroid hydroxylations [33], could replace the NADPH-regenerating system normally used. The concentration of cumene hydroperoxide routinely used to measure benzo(*a*)pyrene hydroxylase was 2.4 mM since it was found that any increase in concentration above this resulted in a decrease in enzyme activity due to the destruction of the enzyme by cumene hydroperoxide. The values of the $K_m$ and $V$ derived from Lineweaver-Burk plots by least-squares regression analysis are given in Table I. There was an overall increase in the value of the $K_m$ as compared with that for the NADPH-supported reaction, which may have been due to the effect of cumene hydroperoxide on the enzyme. Since higher concentrations of cumene hydroperoxide caused destruction of the enzyme activity it may be that at lower concentrations it reacts with the enzyme to modify chemically the active site or to produce conformational changes in the protein which alter the $K_m$ without destroying activity.

Interestingly, the values of $V$ were greater than those for the NADPH-supported benzo(*a*)pyrene hydroxylase by a factor of up to seventeen times.

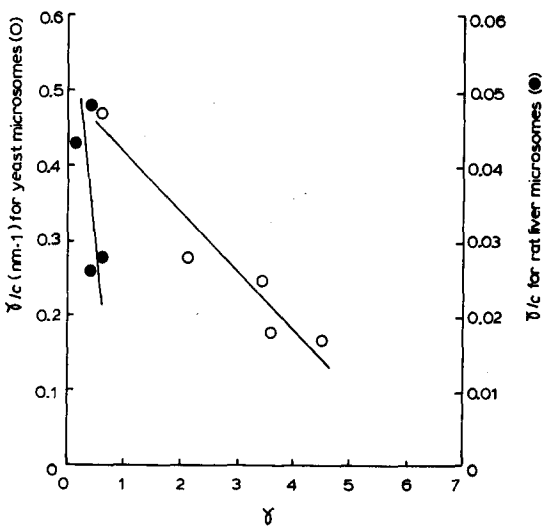


Fig. 3. Scatchard plot of the equilibrium gel filtration of the benzo(*a*)pyrene-cytochrome *P*-450 complex from yeast and rat liver microsomes.

Cumene hydroperoxide bypasses the electron transport chain which must be used when NADPH is the cofactor, and this may increase the overall efficiency of the reaction.

*(iii) Solubilised and immobilised benzo(a)pyrene hydroxylase.* Solubilised enzyme was immobilised onto microcrystalline cellulose as described above and the cellulose was resuspended in 10 ml of 0.1 M Tris/HCl buffer, pH 7.0, containing 20% glycerol. No activity could be detected using NADPH as cofactor, which suggests that the electron transport system necessary for NADPH utilisation was disrupted during the solubilisation procedure. Therefore, $K_m$ and $V$ were determined using cumene hydroperoxide.

The value of the $K_m$ of the solubilised enzyme (0.11 mM) was much less than that of the same enzyme when bound to the microsomal membrane. (Table I). Immobilisation of the enzyme resulted in an increase in the $K_m$ (to 0.20 mM) but not up to the value of the membrane-bound enzyme. This probably reflects the relative degree of steric hindrance imposed onto the active site between the membrane-bound and immobilised benzo(*a*)pyrene hydroxylases, in addition to diffusion limitations.

The solubilised benzo(*a*)pyrene hydroxylase had a smaller $V$ (56.2 pmol 3-hydroxybenzo(*a*)pyrene formed/nmol cytochrome *P*-450 per h) than the membrane-bound enzyme. This probably resulted from conformational changes produced in the enzyme during the solubilisation process. Subsequent immobilisation increased the amount of diffusion limitation and perhaps conformational change, and thus brought about a further decrease in $V$ (to 39.4). The use of glutaraldehyde, for example, may have produced cross-links not only between the enzyme and its support but also within the enzyme itself.

*Spectral interaction of benzo(a)pyrene and cytochrome P-450*

We have previously published the binding spectrum of benzo(*a*)pyrene with yeast cytochrome *P*-450 [34] and Estabrook et al. [35] have demonstrated the same spectrum using rat liver cytochrome *P*-450. The spectrum is essentially the same as a normal type I spectrum but has an additional peak at 360 nm (Fig. 2).

Table II gives the values of the spectral dissociation constants derived from double-reciprocal plots of $A_{415nm} + A_{385-375nm}$ vs. benzo(*a*)pyrene concentration using yeast microsomal fraction, solubilised yeast microsomes and rat liver microsomes. The experiment was also performed using benzo(*e*)pyrene instead of benzo(*a*)pyrene. Solubilisation of the yeast microsomal fraction did

TABLE II

Values of the spectral dissociation constants ($\mu$M) for the binding of benzo(*a*)pyrene and benzo(*e*)pyrene with various microsomal fractions.

| Microsomal fraction | Compound | $K_s$ | Correlation coefficient |
|---|---|---|---|
| Yeast microsomes | Benzo(*a*)pyrene | 32 | 0.999 |
| Solubilised yeast microsomes | Benzo(*a*)pyrene | 55 | 0.998 |
| Rat liver microsomes | Benzo(*a*)pyrene | 9 | 0.978 |
| Yeast microsomes | Benzo(*e*)pyrene | 49 | 0.982 |

not alter the binding spectrum obtained with benzo(*a*)pyrene, nor was there much change in the value of the dissociation constant. Whereas solubilisation had a large effect on the kinetics of benzo(*a*)pyrene metabolism, the effect on the binding to the enzyme was small. This suggests that the interaction of benzo(*a*)pyrene with cytochrome *P*-450 is sufficiently strong so that it occurs to the same extent whether the enzyme is free or held in the microsomal membrane.

The value of the $K_s$ was much lower using rat liver microsomes and probably reflects the much lower values of the $K_m$ for the liver enzyme compared with the yeast enzyme. A close correlation between $K_s$ and $K_m$ values has been found for some type I interactions; Ullrich et al. [36] for example, found that in the case of the O-deethylation of 7-ethoxycoumarin the $K_s$ and $K_m$ values agreed exactly for sodium phenobarbitone- and 3-methylcholanthrene-pretreated animals, but there have also been a number of cases where $K_s$ and $K_m$ values have not agreed. Kratz and Staudinger [37] found that the $K_s$ value for coumarin was some 100 times greater than the $K_m$. Since $K_m$ values for microsomal drug metabolism encompass the individual kinetics of several components of a complex system, the poor correlation between $K_s$ and $K_m$ is not surprising, especially as the optical changes defined by $K_s$ values result primarily from conformational changes in the cytochrome *P*-450, which is only one component of the monooxygenase system.

Benzo(*e*)pyrene gave the same binding spectrum as benzo(*a*)pyrene with yeast microsomes, and the $K_s$ was similar to that of benzo(*a*)pyrene. These results suggest that both these compounds meet the structural requirements for this interaction.

Values of the extinction coefficient of the benzo(*a*)pyrene-cytochrome *P*-450 complex have been published and these make it possible to calculate the percentage of the cytochrome *P*-450 bound to benzo(*a*)pyrene, from the binding spectrum. Estabrook et al. [35] determined a value of 57 $mM^{-1} \cdot cm^{-1}$ for the maximal change in absorbance between 415 and 500 nm, while Cinti et al. [38] used a value of 126 $mM^{-1} \cdot cm^{-1}$ for the change between 385 and 419 nm. Table III shows the percentages of the cytochrome *P*-450 present bound as cytochrome *P*-450-benzo(*a*)pyrene complex and it can be seen that whereas some 30% is bound in the case of the yeast enzyme, only 5% was bound using

TABLE III

The percentage of the cytochrome *P*-450 bound as cytochrome *P*-450-benzo(*a*)pyrene complex for yeast and rat liver microsomes (from spectral data). The actual peak of yeast at 449 nm, rather than 450 or 448 nm as found in mammalian systems.

| Source of enzyme | Wavelength (nm) | Complex concn. (nM) | Cytochrome *P*-450 concn. (nM) | % *P*-450 bound |
|---|---|---|---|---|
| Yeast | 415—500 | 2.7 | 8.3 | 33 |
| | 385—419 | 2.0 | 8.3 | 24 |
| Liver | 415—500 | 0.9 | 18.7 | 5 |
| | 385—419 | 0.7 | 18.7 | 4 |

TABLE IV

Data from the equilibrium gel filtration of the benzo(*a*)pyrene-cytochrome *P*-450 complex for yeast microsomes. *c* is concentration of benzo(*a*)pyrene and *e* is the quantity of cytochrome *P*-450. Thus $K$ = 0.077 $nM^{-1}$ and $n$ = 6.3.

| *c* (nM) | *e* (nmol) | *p* (nmol) | *p/e* ($\gamma$) | $\gamma/c$ ($nM^{-1}$) |
|---|---|---|---|---|
| 1.25 | 0.02 | 0.0117 | 0.59 | 0.472 |
| 7.59 | 0.02 | 0.0422 | 2.11 | 0.278 |
| 13.93 | 0.02 | 0.0690 | 3.45 | 0.248 |
| 20.27 | 0.02 | 0.0720 | 3.60 | 0.178 |
| 26.62 | 0.02 | 0.0900 | 4.50 | 0.169 |

the enzyme from rat liver. Estabrook et al. [35] have shown that after 3-methylcholanthrene-pretreatment of rat, approx. 46% of the enzyme forms a benzo(*a*)pyrene complex, reflecting an increased proportion of cytochrome *P*-450 specific for benzo(*a*)pyrene.

*Equilibrium gel filtration of the benzo(a)pyrene-cytochrome P-450 complex*

Gel filtration experiments were carried out at increasing benzo(*a*)pyrene concentrations (*c*) using the same amount of cytochrome *P*-450 (*e*). The specific activity of the benzo(*a*)pyrene was determined by counting a 50 $\mu$l sample of the equilibrating buffer, and this was used to calculate the amount of benzo(*a*)pyrene bound to the cytochrome *P*-450 (*p*). From the values of *p*, *e* and *c* thus determined, *p/e* ($\gamma$) and $\gamma/c$ were calculated and Scatchard plots were constructed (Fig. 3). The values of the number of binding sites ($n$) and the apparent association constant ($K$) were then estimated by extrapolation. The experiment was performed using yeast microsomes (Table IV) and control rat liver microsomes (Table V).

The values of $n$ determined were 6.3 for yeast microsomes and 0.95 for rat liver microsomes; thus it would seem that 6 mol of benzo(*a*)pyrene were bound to each mol of yeast cytochrome *P*-450 present, compared with 1 mol of benzo(*a*)pyrene for rat liver cytochrome *P*-450. These values are based on the assumption that the binding sites of the enzyme are all occupied. However, we have shown that in the case of the yeast enzyme only 30% of the enzyme formed the benzo(*a*)pyrene-cytochrome *P*-450 complex. This means that in order to obtain a stoicheiometry of 6 : 1, the total number of binding sites in

TABLE V

Data from the equilibrium gel filtration of the benzo(*a*)pyrene-cytochrome *P*-450 complex for rat liver microsomes. *c* is concentration of benzo(*a*)pyrene and *e* is the quantity of cytochrome *P*-450. Thus $K$ = 0.064 $nM^{-1}$ and $n$ = 0.95.

| *c* (nM) | *e* (pmol) | *p* (pmol) | *p/e* ($\gamma$) | $\gamma/c$ ($nM^{-1}$) |
|---|---|---|---|---|
| 2.5 | 3.2 | 0.35 | 0.108 | 0.043 |
| 8.8 | 3.2 | 1.37 | 0.428 | 0.048 |
| 15.2 | 3.2 | 1.28 | 0.400 | 0.026 |
| 21.5 | 3.2 | 1.90 | 0.594 | 0.028 |

the 30% of the cytochrome *P*-450 which binds benzo(*a*)pyrene would have to be 20. Similarly, to obtain a 1 : 1 ratio for rat liver cytochrome *P*-450 of which only 5% forms a complex with benzo(*a*)pyrene, there would also have to be 20 binding sites, including membrane binding sites, in both cases.

The values obtained for the association constants of the yeast and rat liver enzymes were 0.077 $nM^{-1}$ and 0.064 $nM^{-1}$ (Tables IV and V). This similarity suggests that the affinity with which the benzo(*a*)pyrene is bound to the binding sites of these enzymes is approximately the same. These association constants are equivalent to dissociation constants of approx. $10^{-8}$ M, which is smaller than the values obtained for the spectral dissociation constants and the Michaelis constants.

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