Biochemical Pharmacology, Vol. 19, pp. 1449–1455. Pergamon Press. 1970. Printed in Great Britain

STUDIES ON THE HYDROXYLATION OF 3,4-BENZPYRENE BY HEPATIC MICROSOMES

EFFECT OF ALBUMIN ON THE RATE OF HYDROXYLATION OF 3,4-BENZPYRENE*

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(*Received* 7 *July* 1969; *accepted* 5 *September* 1969)

Abstract—The enzyme system which hydroxylates 3,4-benzpyrene was studied using 9000 *g* supernatant and microsomes from livers of untreated rats and 3-methylcholanthrene-treated rats. When 9000 *g* supernatant obtained from less than 10 mg of liver is used as the enzyme source, the rate of formation of hydroxy-3,4-benzpyrene was proportional to tissue concentration. In contrast, when microsomes obtained from less than 10 mg of liver were used, the rate of reaction was not linear with tissue concentration. However, linearity could be obtained if albumin was added to the reaction mixture. The kinetic constants for 3,4-benzpyrene hydroxylase obtained with liver microsomes, in the presence of albumin, were similar to those previously reported using 9000 *g* supernatant in the absence of albumin. Evidence is presented which shows that the addition of albumin or liver microsomes to the incubation mixture used for the assay of 3,4-benzpyrene hydroxylase activity resulted in an increase in the amount of 3,4-benzpyrene solubilized.

THE CARCINOGENIC polycyclic hydrocarbon 3,4-benzpyrene is hydroxylated in the liver of various species including man.[1–3] Conney *et al.*[1] first showed that 3,4-benzpyrene hydroxylase was localized in liver microsomes, required NADPH and oxygen for activity, and was increased when animals were treated with 3,4-benzpyrene or other polycyclic hydrocarbons. Since then this substrate has been widely used in test systems to reveal induction by the polycyclic hydrocarbons. The advantage of using 3,4-benzpyrene as a model "hydrocarbon inducible" substrate is that small amounts of tissue can be used in the assay system since the product of the hydroxylation reaction, hydroxy-3,4-benzpyrene, is very fluorescent and, thereby, small amounts of product and initial reaction rates can be determined.

During the course of our studies with this substrate, it was found that when 9000 *g* supernatant obtained from 2 to 10 mg of liver was used as the source of the hydroxylase, the rate of formation of hydroxy-3,4-benzpyrene was proportional to the tissue concentration. In contrast, when microsomes obtained from less than 10 mg of liver were used, the rate of reaction was not linear with tissue concentration. This problem was investigated further and the results of our studies are presented in this paper. The results show that when microsomes obtained from small amounts of liver are used to study 3,4-benzpyrene hydroxylation, linearity of reaction rate can be obtained if

* This research was supported in part by Research Contract No. PH 43-65-1066 from the Pharmacology–Toxicology Programs, NIGMS, NIH.

albumin is added to the reaction mixture. In addition, studies presented in this paper show that the addition of albumin to the reaction mixture results in an increase in the amount of 3,4-benzpyrene solubilized. The results presented in this paper also show that the apparent Michaelis constant (K_m) for 3,4-benzpyrene hydroxylase obtained with microsomes was decreased after 3-methylcholanthrene administration to rats. This is similar to the effect of 3-methylcholanthrene on 3,4-benzpyrene hydroxylase previously reported[4] using 9000 *g* supernatant or whole homogenate in the absence of albumin.

METHODS

Male Sprague–Dawley rats weighing 130–150 g were used. Food and water were supplied *ad lib.* Rats receiving 3-methylcholanthrene were given the polycyclic hydrocarbon dissolved in corn oil at a dose of 20 mg per kg by a single i.p. injection. Animals were killed 24 hr later. 3,4-Benzpyrene was obtained from K and K Laboratories, Fairview, N. Y. Crystalline bovine serum albumin was obtained from Sigma Chemical Co.

Isolation of microsomes. Rats were killed by decapitation and their livers were homogenized in 4 vol. of 1·15% KCl with a Dounce homogenizer. The homogenates were centrifuged for 20 min at 9000 $g_{(av.)}$ and microsomes were isolated by centrifugation of the 9000 *g* supernatant at 105,000 $g_{(av.)}$ for 60 min in a Spinco model L-2 ultracentrifuge using a Ti 50 rotor. The microsomal pellet was suspended in 1·15% KCl solution so that each milliliter of the suspension contained the equivalent of 250 mg of liver wet weight. In untreated rats, the 9000 *g* supernatant and the microsomal suspensions obtained from 1 g of liver wet weight contained on an average 110·4 mg and 29·6 mg of protein respectively. In rats treated with a single dose of 3-methylcholanthrene and killed 24 hr later, the 9000 *g* supernatant and the microsomal suspensions obtained from 1 g of liver wet weight contained on an average 112·5 mg and 32·1 mg of protein respectively. Protein was determined by the method of Sutherland *et al.*[5] Varying concentrations of 9000 $g_{(av.)}$ supernatant or 105,000 *g* microsomal suspensions were prepared by diluting the different preparations with isotonic KCl. The temperature during all the above steps was 4°.

3,4-*Benzpyrene metabolism.* 3,4-Benzpyrene hydroxylase activity was determined as previously described.[2, 6] Prior to the addition of the substrate, the contents of the incubation flasks were equilibrated at 37° for 1 min. After addition of 3,4-benzpyrene the flasks were incubated at 37° for an additional 5 min and then assayed for the hydroxylated metabolites. The reaction rate was linear for at least 5 min at 37°. The activation and fluorescence spectra of the hydroxylated metabolites formed by liver are identical to that of 8-hydroxy-3,4-benzpyrene;[1] therefore, the latter compound was used as a standard to calculate the amount of hydroxy-3,4-benzpyrene formed. All enzyme kinetic data were plotted according to the method of Lineweaver and Burk[7] and were analyzed statistically by use of the Student's *t*-test.[8]

Solubility studies. ^{3}H-3,4-Benzpyrene (Nuclear Chicago Corp., Des Plaines, Ill.) was diluted with nonradioactive 3,4-benzpyrene to give a specific activity of 14·5 μc per mg. Varying amounts of either albumin dissolved in H_2O or microsomes from untreated or 3-methylcholanthrene-treated rats suspended in 1·15% KCl were added to the 3·1-ml incubation mixture described previously.[2, 6] After the addition of 50 μg of tritiated benzpyrene, the contents of the incubation flasks were incubated at 37°

for 5 min. The incubation of 50 μg of 3,4-benzpyrene with microsomes and cofactors for 5 min at 37° resulted in the metabolism of less than 1 per cent of the substrate. The incubation mixture was cooled to 4° and centrifuged for 20 min at 4000 $g_{(av.)}$ to precipitate the undissolved 3,4-benzpyrene, and a 0·1-ml aliquot of the supernatant liquid was added to 10 ml of the scintillation mixture of Bray[9] and quantified in a liquid scintillation spectrometer. The recovery of 3,4-benzpyrene in the 4000 *g* supernatant was used as an estimate of the solubilized 3,4-benzpyrene. It is recognized that the radioactivity in the 4000 *g* supernatant may not be in true solution and may be bound to albumin and other proteins in microsomes.

RESULTS

Effect of varying tissue concentration on rate of hydroxylation of 3,4-*benzpyrene.* Varying concentrations of 9000 *g* supernatant, microsomes suspended in 1·15% KCl, or microsomes suspended in equivalent amounts of the 100,000 *g* soluble fraction (reconstituted 9000 *g* supernatant) were incubated for 5 min at 37° in the presence of 50 μg of 3,4-benzpyrene and an NADPH generating system.[2, 6] The formation of hydroxy-3,4-benzpyrene was measured and the plot of activity vs. enzyme concentration is presented in Figs. 1 and 2. When 9000 *g* supernatant or reconstituted 9000 *g* supernatant was used as the enzyme source, the formation of hydroxy-3,4-benzpyrene was

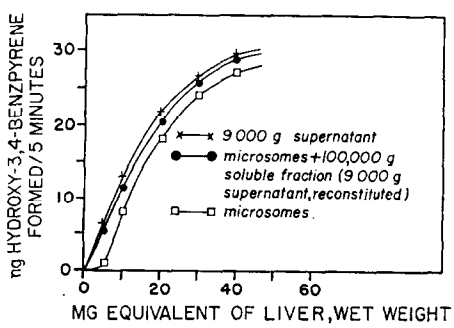

FIG. 1. Effect of tissue concentration on the rate of formation of hydroxy-3,4-benzpyrene. 9000 *g* supernatent, or microsomes suspended in equivalent amounts of soluble fraction, or microsomes suspended in 1·15% KCl obtained from varying amounts of liver, were incubated with 50 μg 3,4-benzpyrene and NADPH generating system[2, 6] at 37° for 5 min in a total volume of 3·1 ml.

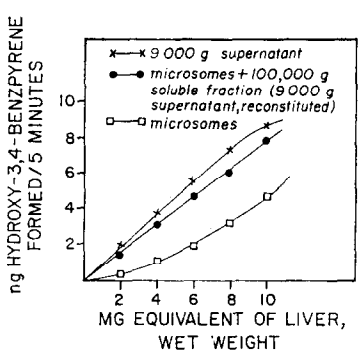

FIG. 2. Effect of low tissue concentrations on the rate of formation of hydroxy-3,4-benzpyrene. Conditions of the experiment are the same as described in the legend of Fig. 1.

linear with tissue concentration when less than 10 mg of liver wet weight was used. At concentrations greater than 10 mg, linearity was no longer maintained. However, when microsomes obtained from 2 to 40 mg of liver were used as the source of the enzyme, the reaction rate was not linear at any concentration of microsomes (Figs. 1 and 2).

Effect of albumin on the hydroxylation of 3,4-*benzpyrene by the microsomal fraction of liver.* Microsomal suspensions in 1·15% KCl from untreated and 3-methylcholanthrene-treated rats were prepared. Microsomes from various amounts of liver were incubated with 50 μg of 3,4-benzpyrene and an NADPH generating system[2, 6] in the absence or presence of 0·6 mg of albumin. The formation of hydroxy-3,4-benzpyrene was measured, and the results are presented in Fig. 3. With no albumin in

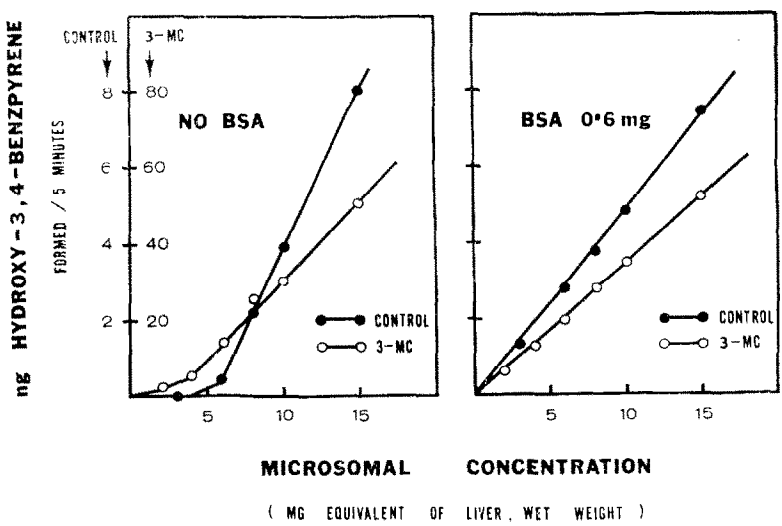

FIG. 3. Effect of addition of albumin on the formation of hydroxy-3,4-benzpyrene after incubation of microsomes with 3,4-benzpyrene. Microsomes, from varying amounts of liver obtained from untreated (——) or 3-methylcholanthrene (3-MC)-treated (– – –) rats, were incubated with 50 μg of 3,4-benzpyrene in the absence of and in the presence of 0·6 mg of albumin (BSA), using the cofactor mix and assay procedures as described in Methods. Values on the left hand and right hand side of the ordinate represent ng hydroxy-3,4-benzpyrene formed per 5 min using microsomes from untreated and 3-MC-treated rats respectively.

the incubation mixture, the reaction rate was not linear with tissue concentration. However, in the presence of 0·4 or 0·6 mg of albumin, the rate was proportional to the enzyme concentration.

Kinetics of 3,4-*benzpyrene hydroxylation by microsomes from untreated and* 3-*methylcholanthrene-treated rats.* Microsomes from 4 mg of liver wet weight obtained from untreated rats or rats to which 3-methylcholanthrene was given were incubated with various amounts of 3,4-benzpyrene, 0·6 mg of albumin, and an NADPH generating system as previously described,[2, 6] and the rate of formation of hydroxy-3,4-benzpyrene was determined. The data, as plotted according to the method of Lineweaver and Burk,[7] can be seen in Fig. 4. The apparent K_m for the hydroxylation of 3,4-

benzpyrene by the microsomal hydroxylase from untreated rats ($0{\cdot}86 \times 10^{-5}$ M) was significantly different ($P < 0{\cdot}01$) than the apparent K_m value ($0{\cdot}24 \times 10^{-5}$ M) obtained for the hydroxylase from 3-methylcholanthrene-treated rats. The maximal velocity (V_{max}) was increased about 3-fold after a single injection of 3-methylcholanthrene.

Increase in amount of 3,4-*benzpyrene in solution in the presence of albumin or liver microsomes.* Varying concentrations of albumin were added to an incubation mixture[2, 6] containing 50 μg of ^{3}H-3,4-benzpyrene and no microsomes. The incubation mixture was allowed to stand for 5 min at 37° and then was centrifuged at 4000 *g*

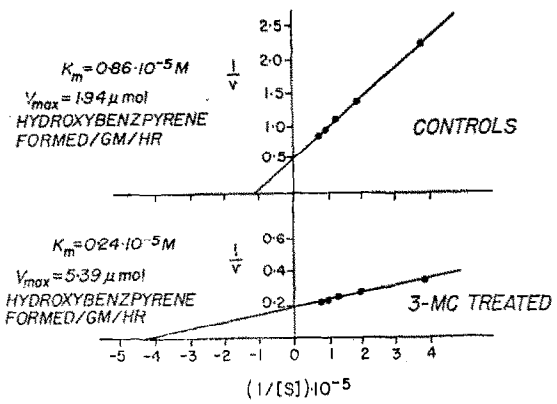

FIG. 4. Plot, 1/v vs. 1/s, for the hydroxylation of 3,4-benzpyrene by rat liver microsomes. Velocities are given as μmoles hydroxy 3,4-benzpyrene formed per g liver per hr; substrate concentrations are in moles per liter. Each point represents the mean value of five different experiments. Each experiment was done with a different rat.

for 20 min to precipitate the undissolved 3,4-benzpyrene. The recovery of radioactivity in 0·1 ml of the clear supernatant was then quantified in a liquid scintillation spectrometer. With the addition of 0·1 mg albumin, the recovery of the polycyclic hydrocarbon in the supernatant increased markedly and the greatest solubilization occurred when 0·6 mg of albumin was added to the incubation mixture (Fig. 5). The addition of 0·6 mg albumin resulted in the solubilization of 56 per cent of the tritiated 3,4-benzpyrene. Similarly, when microsomes instead of albumin were added, the recovery of 3,4-benzpyrene in the supernatant increased (Fig. 6). Maximum recovery of radioactivity in the supernatant was obtained when microsomes from 8 to 10 mg of liver wet weight were added to the incubation mixture. The addition of microsomes from 3-methylcholanthrene-treated rats increased the recovery of 3,4-benzpyrene to the same extent as did the addition of microsomes from normal rats (Fig. 6). The dashed line in Fig. 6 represents the amount of ^{3}H-3,4-benzpyrene recovered in the supernatant when 0·6 mg of albumin, instead of microsomes, was added to the incubation mixture. If the cofactors and buffers used for hydroxylase reaction[2, 6] were replaced by distilled water, the recovery of 3,4-benzpyrene in the supernatant was not increased by albumin. However, to obtain comparable recovery in the presence of albumin, the cofactor mixture could be replaced by 0·05 M, pH 7·4, Tris buffer, or 0·1 M, pH 7·4, K_2HPO_4–KH_2PO_4 buffer.

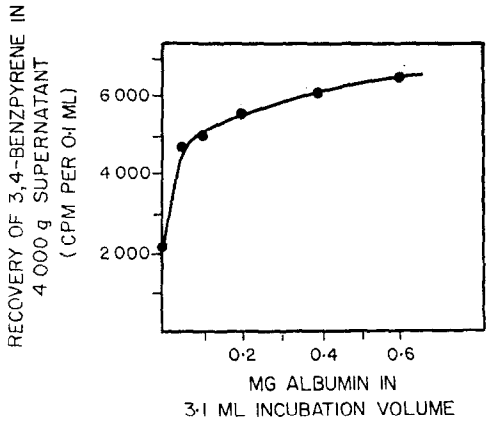

FIG. 5. Effect of albumin on the amount of 3,4-benzpyrene dissolved in 4000 *g* supernatant. Varying concentrations of albumin were incubated with 50 μg of ^{3}H-3,4-benzpyrene (12,000 cpm/0·1 ml incubation mixture) at 37° for 5 min. Contents of incubation flask were centrifuged at 4000 *g* for 20 min and 0·1 ml of clear supernatant was counted.

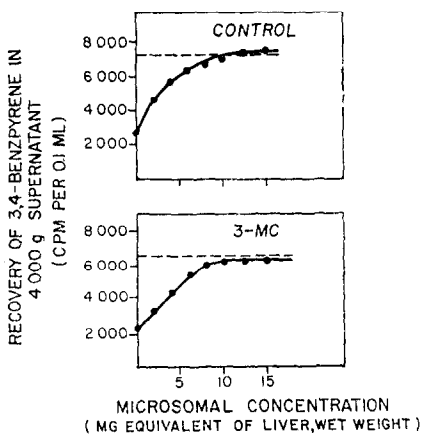

FIG. 6. Recovery of 3,4-benzpyrene in the presence of microsomes obtained from varying amounts of liver. Microsomes from varying amounts of liver from untreated or 3-methylcholanthrene-(3-MC) treated rats were incubated with 50 μg of ^{3}H-3,4-benzpyrene (12,000 cpm/0·1 ml incubation mixture) at 37° for 5 min. Contents of the incubation flask were centrifuged at 4000 *g* for 20 min and 0·1 ml of supernatant was counted (straight line). Dashed line represents the amount of 3,4-benzpyrene recovered when 0·6 mg of albumin, instead of microsomes, was used in the incubation mixture.

DISCUSSION

The advantage of using 3,4-benzpyrene as a substrate to determine liver microsomal enzyme activity is the small amount of tissue which can be used because of the highly fluorescent nature of the products formed. The method, thus, allows the determination of enzyme activity in small samples of tissue obtained from human biopsy. Results presented in this paper show that precautions have to be taken when very small tissue concentrations are used. The reaction rate was linear with low enzyme concentrations when the 9000 *g* supernatant was used as the enzyme source. When liver microsomes equivalent to less than 10 mg of liver were used as the enzyme source, the rate of

hydroxylation was not proportional to the enzyme concentration. However, linearity of reaction rate could be obtained with microsomes from small amounts of liver, if 0·4 to 0·6 mg of bovine serum albumin was added to the incubation flask.

Evidence presented in this paper indicates that albumin or a high concentration of microsomes can solubilize 3,4-benzpyrene. The amount of dissolved 3,4-benzpyrene increased when increasing amounts of microsomes or albumin were added to the incubation mixture. In the absence of albumin, the amount of 3,4-benzpyrene in solution varies with the microsomal concentration and, therefore, linearity of hydroxylation rate cannot be obtained. In the presence of albumin, however, the concentration of dissolved 3,4-benzpyrene remains constant as the tissue concentration is increased and linearity of the reaction rate is obtained. Although albumin probably acts by increasing the solubility of 3,4-benzpyrene, the possibility that an inhibitor, such as a heavy metal, in the cofactor mixture inhibits the hydroxylase at low enzyme concentrations cannot be excluded. Addition of albumin could bind this inhibitor and prevent its access to enzymic sites in the microsomes.

The apparent K_m values obtained for 3,4-benzpyrene hydroxylase, using microsomes from untreated or 3-methylcholanthrene-treated rats, were similar to those reported previously using 9000 *g* supernatant in the absence of albumin.[4] The change in the apparent K_m for the hydroxylation of 3,4-benzpyrene in 3-methylcholanthrene-treated rats has been reported previously.[4] It should be emphasized that the K_m values reported in this paper are apparent K_m values and may be influenced by nonspecific binding[10] and by the transport of the substrate to the enzyme.

The results presented with albumin in this study may be important in the study of the metabolism of other drugs, since whenever sensitive methods for the assay of product and good substrate affinity for enzyme exists, solubilization of lipid soluble substrates may be enhanced by the addition of protein. In most methods available today for assaying drug metabolism, the problem discussed in this paper does not occur because the assay methods are not sensitive and, therefore, large amounts of tissue are used in the incubation medium and the high tissue concentrations can supply the necessary protein for solubilizing the drugs.

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