Studies on the Enzymic Hydroxylation of 3,4-Benzpyrene

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SUMMARY

We have investigated the relationship between the capacity of rat liver microsomal preparations to metabolize several foreign substances, and their content of the carbon monoxide-binding pigment, P-450. A rapid and convenient radioactive assay for the hydroxylation of 3,4-benzpyrene has been developed and the kinetics of this system studied. Optically clear, but not soluble, enzyme preparations were obtained by treatment of rat liver microsomes derived from benzpyrene-treated animals with the nonionic detergent Triton N-101. It was shown that the capacity of these preparations to hydroxylate 3,4-benzpyrene after various types of treatments, was impaired in direct proportion to the conversion of P-450 to its inactive form, P-420. Benzpyrene hydroxylase activity and the content of P-450 during storage were conserved to a large degree by the addition of glycerol to the medium. Treatment of rats with either 3,4-benzpyrene or phenobarbital resulted in elevated levels of benzpyrene hydroxylase, zoxazolamine hydroxylase, and *p*-nitroanisole demethylase activities as well as in a higher P-450 content, although the increases of these various components resulting from drug treatment were not in the same proportion. Evidence for the reactivation of acid-treated enzyme preparations by the addition of flavins was obtained.

INTRODUCTION

The powerfully carcinogenic polycyclic hydrocarbon, 3,4-benzpyrene,[1] undergoes a variety of hydroxylation reactions in the livers of susceptible species. Ten years ago, Conney *et al.* (1, 2) established that these metabolic reactions in the rat were localized in liver microsomes and exhibited a requirement for reduced triphosphopyridine nucleotide and oxygen. A number of major metabolites (8-hydroxy- and 10-hydroxybenzpyrene) and minor metabolites (5,8-dihydroxybenzpyrene, as well as 5,8- and 5,10-benzpyrenequinones) were isolated, and these were shown to be identical with the products isolated from animals to whom benzpyrene had been administered (2). Conney *et al.* (2) further described the remarkable and now widely recognized induction of benzpyrene hydroxylase activity by the prior administration of small doses of benzpyrene itself, 3-methylcholanthrene, or other polycyclic hydrocarbons. Subsequent studies have shown that similar inductions of the microsomal drug-metabolizing enzyme systems are evoked by administration of barbiturates, steroids, insecticides, or other drugs (3–11). These inductions are associated with increases in the relative quantity of the smooth endoplasmic reticulum (12, 13) and of the concentration of a hemoprotein pigment, capable of reacting with carbon monoxide, which has been designated as P-450, and is an essential component of many TPNH-dependent hydroxylation reactions occurring in both microsomes and mitochondria (10, 14–19).

A detailed understanding of these hydroxylation reactions and of the oxygen-

[1] The hydrocarbon 3,4-benzpyrene is referred to as benzpyrene in this paper.

and substrate-activating proteins concerned will ultimately require fractionation of the various drug-metabolizing reactions. While numerous attempts to solubilize hepatic microsomal hydroxylation systems have met with failure (20, 21), this feat has now been partially accomplished in the case of the steroid 11β-hydroxylation of the mitochondria of the beef adrenal cortex (22).

Microsomes subjected to treatment with detergents, phospholipases or proteolytic enzymes exhibit little or no hydroxylase activity, although Narasimhulu (23) has been able to obtain optically clear preparations which retain considerable steroid 21-hydroxylase activity after treatment of bovine adrenalcortical microsomes with the nonionic detergents Triton X-100 and Triton N-101. It has also been found that the hemoprotein P-450 of preparations treated with deoxycholate or phospholipases is converted to a soluble, but altered, form of the pigment (P-420) which combines with carbon monoxide to give a reduced difference spectrum displaying an absorption peak at 420 mμ (14, 24).

This paper describes the preparation of a relatively stable and optically clear enzyme system from rat liver microsomes catalyzing the hydroxylation of benzpyrene. A simple radioactive assay for the hydroxylation reaction has been developed, and some of the kinetic aspects of the system have been examined. The increases in activity of this enzyme system induced by administration of phenobarbital and benzpyrene bear a close relation to the content of the hemoprotein P-450. Efforts have also been made to fractionate this multienzyme system and to delineate its properties.

MATERIALS AND METHODS

Benzpyrene was obtained from Calbiochem and was purified by vacuum sublimation. Nonspecifically tritiated benzpyrene (1.7 C/mmole) was supplied by Nuclear Chicago Corporation; it was diluted to a specific activity of 0.1 mC/mmole with nonradioactive benzpyrene. Male Sprague-Dawley rats purchased from Huntington Farms, Conshohocken, Pennsylvania, were used in all experiments. P-L Biochemicals supplied TPN. Glucose 6-phosphate (sodium salt) and purified yeast glucose 6-phosphate dehydrogenase were obtained from Sigma and Boehringer-Mannheim, respectively. Triton N-101 was supplied by Rohm and Haas. *Trimeresurus flavoviridis* snake venom was purchased from Sigma. Matheson Scientific Company supplied the carbon monoxide. All other materials were of the highest quality available commercially.

Preparation of microsomes. Hepatic microsomes were prepared by homogenization of livers from decapitated rats in 2–4 volumes of ice-cold medium containing 0.25 M sucrose and 0.05 M potassium phosphate buffer or Tris-chloride buffer at pH 7.4. The homogenization was performed in a Waring Blendor operated at one-half maximum speed for 1 min. The enzyme preparations used for most of the experiments were obtained from male rats weighing 250–300 g that had received intraperitoneal injections of benzpyrene in sesame oil (30 mg/kg) 24 hr before they were killed, in order to elevate the benzpyrene hydroxylase activity.

Enzyme induction was studied in recently weaned rats weighing 50–75 g. The animals were treated by intraperitoneal injection with either aqueous sodium phenobarbital (50 mg/kg) daily for 4 days or with benzpyrene in sesame oil (30 mg/kg) 24 hr prior to preparation of the microsomes.

The homogenate was centrifuged at 20,000 *g* for 15 min, and the supernatant, after filtration through glass wool, was centrifuged at 105,000 *g* for 90 min. All these operations were carried out at 0–5°. The pellets were suspended in the same buffer at pH 7.4 by means of a few manual strokes in a glass homogenizer, and were again centrifuged at 105,000 *g* for 90 min. This washing procedure was repeated, and the pellet was suspended in buffer to a final concentration equivalent to 1 g of tissue per milliliter. Such preparations were found to contain about 10–15 mg protein per milliliter when analyzed by the method of Lowry *et al.* (25).

Preparation of Triton extracts. To 4 vol-

umes of microsomal suspension was added 1 volume of 10% aqueous Triton N-101 containing 0.05 M buffer at pH 7.4, to give a final concentration of 2% Triton. The mixture was stirred for 1 hour at 0°, and then centrifuged for 90 min at 105,000 *g*. The supernatant fluid was clear and brownish yellow in color. The precipitate was composed of a tightly packed colorless pellet, probably consisting of both glycogen and ribosomes, on top of which lay an additional reddish brown layer.

The detergent, which is not dialyzable, could be removed almost entirely from the high speed supernatant fraction by the addition of ethanol at —15° to a final concentration of 33% by volume. When this material was centrifuged at 10,000 *g* for 15 min, 80–90% of the protein was precipitated, leaving the ethanol-soluble Triton in the solution. The precipitate, after suspension in buffer yielded a deep yellow transparent fluid hereinafter referred to as the "33% ethanol fraction." This fraction was then dialyzed overnight against buffer at pH 7.4. Although optically clear, this material was not a solubilized preparation of microsomes, since further attempts at purification by ammonium sulfate fractionation, gel filtration, or ion exchange chromatography proved fruitless. Furthermore, although there was almost no precipitation of the material after centrifugation for 1 hr at 105,000 *g*, complete precipitation of all protein and colored matter occurred after 12 hr of centrifugation under these conditions.

Assay procedures. The metabolism of benzpyrene by the cell-free microsomal system was assayed by measuring the decrease in hexane-extractable radioactivity from basic solution, after incubation of ^{3}H-benzpyrene under conditions patterned after Conney *et al.* (2). The reactions were carried out in a Dubnoff metabolic shaker in the presence of air. The vessels were incubated at 37° and the assay was performed in darkness since benzpyrene undergoes photodecomposition. The incubation mixture of 1.5-ml volume contained 16.6 mM potassium phosphate buffer at pH 7.4; 2 mM glucose 6-phosphate; 0.67 mM TPN; 10 μg (approximately 1.5 units) of glucose 6-phosphate dehydrogenase; 33.3 μM ^{3}H-benzpyrene (5000 cpm); and enzyme (microsomes or a derivative thereof). Neither KCl nor nicotinamide (cf. 2) were included as these substances failed to stimulate the reaction and were inhibitory at high concentrations.

The reactions were initiated by the addition of the ^{3}H-benzpyrene in 0.05 ml of ethanol. The incubation was terminated by the addition of 3.5 ml of 0.25 N KOH in 50% ethanol. The reaction mixtures were transferred quantitatively to 50-ml capacity glass-stoppered centrifuge tubes with 10 ml of redistilled hexane and were shaken mechanically for 30 min. The tubes were removed from the shaker and allowed to stand for 10 min to permit separation of the aqueous and organic phases. Five milliliters of the organic phase, which contains the unreacted benzpyrene, were then pipetted into liquid scintillation counting vials together with 10 ml of scintillator solution (4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis [2-(5-phenyloxazolyl)] benzene per liter of toluene). With this assay procedure there was no need to wait for 24 hr (cf. 2) after termination of the reaction to extract the unreacted benzpyrene, since zero time controls and reaction mixtures incubated in the absence of enzyme or with heat-denatured enzyme resulted in good recovery (95–98%) of the ^{3}H-benzpyrene added.

The validity of this rapid and convenient assay procedure is based on the assumptions that (a) the metabolites of benzpyrene which are believed to be phenols and quinones are not extractable from basic solutions by hexane, and (b) benzpyrene does not bind irreversibly to protein before it undergoes hydroxylation. It should be pointed out that several hydroxylation products of benzpyrene are formed and that upon subsequent acidification of the basic solution, only a portion of the residual radioactivity can be extracted from the reaction mixture even with highly polar solvents.

Zoxazolamine hydroxylase was measured by the method of Conney *et al.* (26). A modification of the method of Netter (27,

28) was utilized for the determination of *p*-nitroanisole demethylase activity. The spectrophotometric assay was carried out at 37° and measured the appearance of *p*-nitrophenol at 420 mμ. The 1-ml reaction system contained 75 mM potassium phosphate buffer at pH 7.8, 1 mM TPN, 6 mM glucose 6-phosphate, 20 μg of glucose 6-phosphate dehydrogenase, 0.4 mM *p*-nitroanisole, and microsomes or the "33% ethanol fraction" derived therefrom.

Protein concentrations were determined by the method of Lowry *et al.* (25). Procedures for measuring the concentration of the hemoprotein P-450 and its modified form P-420 are given with the relevant protocols.

RESULTS AND DISCUSSION

The freshly prepared "33% ethanol fraction" exhibited about the same specific activity for hydroxylating benzpyrene as the untreated microsomes. However, the protein concentration of this detergent-treated extract was only about one-half that of microsomes per equivalent weight of original liver tissue; consequently, only 50% of the total activity present in the washed microsomes was recovered in the "33% ethanol fraction." The fractionated preparations metabolized 2–3 mμmoles of benzpyrene per minute per milligram of protein under conditions of the assay.

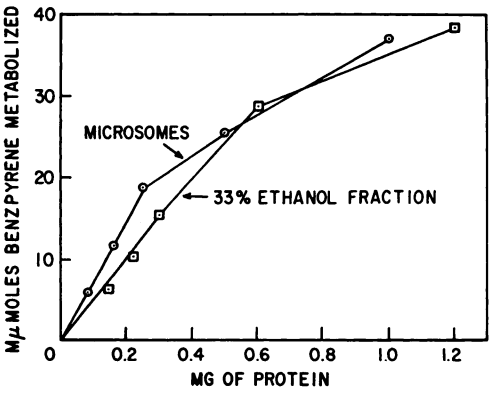

FIG. 1. *Hydroxylation of benzpyrene as a function of enzyme concentration*

The assay conditions are described under Materials and Methods. The quantity of microsomal or "33% ethanol fraction" protein added to the assay system is shown on the abcissa. Incubation time was 20 min.

The hydroxylation of benzpyrene was linear with respect to the quantity of microsomes or "33% ethanol fraction" added, up to concentrations which metabolized 50% of the substrate (Fig. 1). Figure 2 illustrates the amount of benzpyrene metabolized as a function of incubation time.

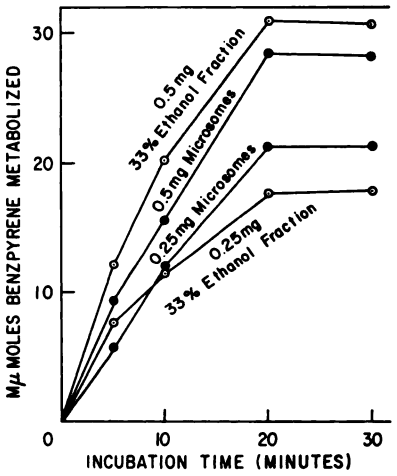

FIG. 2. *Hydroxylation of benzpyrene as a function of incubation time*

The assay conditions are described under Materials and Methods. The time of incubation is shown on the abcissa. The quantity of microsomes or "33% ethanol fraction" added to the assay system was equivalent to 0.25 mg of protein or 0.50 mg of protein, as indicated.

Under the conditions of the assay, the reaction was linear for the first 20 min of incubation, after which a marked decrease in the rate of hydroxylation was observed.

From double reciprocal plots, the Michaelis constant for the hydroxylation of benzpyrene was determined to be 28.5 μM when catalyzed by microsomes and 21.5 μM when catalyzed by the "33% ethanol fraction." Additional experiments showed that these values were independent of enzyme concentration.

Carbon Monoxide Inhibition

Like a number of other oxidation reactions catalyzed by microsomes and requiring TPNH and molecular oxygen (16, 18, 22), the hydroxylation of benzpyrene is

TABLE 1

Inhibition of benzpyrene metabolism by carbon monoxide

The reaction components of the assay system except for the enzyme were introduced into the flasks which were then sealed with tight-fitting silicone stoppers. The flasks were evacuated by means of an aspirator attached to a hypodermic needle inserted through the silicone stoppers. The various gas mixtures shown in the table were then introduced through the same needle. This was accomplished by means of a three-way stopcock which allowed connection of the reaction vessels to either an aspirator or the gas supply. In order to assure complete equilibration of the reaction components with the gaseous phase, the flasks were again evacuated, followed by introduction of the proper gas mixture. This procedure was repeated four times. The flasks were then placed in the Dubnoff shaker, and the enzyme was injected through the stopper by means of a syringe. The enzyme source was the "33% ethanol fraction" (1.2 mg protein), and the flasks were incubated for 30 min.

Experiment No.	Composition of atmosphere	Amount of benzpyrene metabolized (mμmoles)	Relative rates (%)
1	80% N_2:20% O_2	27.0	100
2	90% N_2:10% O_2	26.8	99
3	70% N_2:10% O_2:20% CO	20.0	74
4	10% N_2:10% O_2:80% CO	11.6	43

inhibited by carbon monoxide. In the experiment shown in Table 1, the metabolism of benzpyrene was inhibited by 26% and 57% in the presence of 20% and 80% carbon monoxide, respectively. In these experiments the partial pressure of oxygen was maintained at 10%, which appears to saturate the oxygen-activating system (Table 1).

Relation between Content of P-450 and Hydroxylase Activity

The presence in liver microsomes of a pigment capable of binding carbon monoxide was first described by Klingenberg (29) and by Garfinkel (30). Subsequently, it was shown (14, 15, 31) that this pigment was a hemoprotein, designated P-450, because in the reduced form it combines with carbon monoxide to give a difference spectrum with an absorption peak at 450 mμ. When particulate preparations of P-450 were treated with deoxycholate, or incubated with phospholipase, native P-450 was transformed into a soluble derivative pigment, P-420. Largely due to the investigations of Rosenthal, Cooper, Estabrook, and their co-workers (16, 22), it has now been established that P-450 is an essential component of both microsomal and mitochondrial TPNH-dependent hydroxylation reactions and probably functions as the "oxygen-activating component" of the oxidase system.

As illustrated in Fig. 3, the freshly prepared "33% ethanol fraction" contains nearly the same amount of P-450 per milligram of protein as is found in intact microsomes. In addition, the detergent-treated material contains a small amount of P-420 which is not detectable in the fresh microsomes. However, the P-450 of the "33% ethanol fraction" is far more labile than that of the intact microsomes and undergoes conversion to P-420 when stored at 4° or −20°. Figures 3A and 3C compare the relative stabilities of P-450 in the intact microsomes and the "33% ethanol fraction." This conversion was accompanied by a loss in benzpyrene hydroxylase activity (Table 2). The presence of glycerol in concentrations ranging from 20 to 50% offered considerable protection against the conversion of P-450 to P-420 (Figs. 3B and 3D). In addition, Table 2 illustrates that benzpyrene hydroxylase activity was also conserved, to a large extent, upon storage in the presence of glycerol, especially in the case of the "33% ethanol fraction," in which the activity was rather more unstable than in the intact microsomes. The direct addition of glycerol to preparations

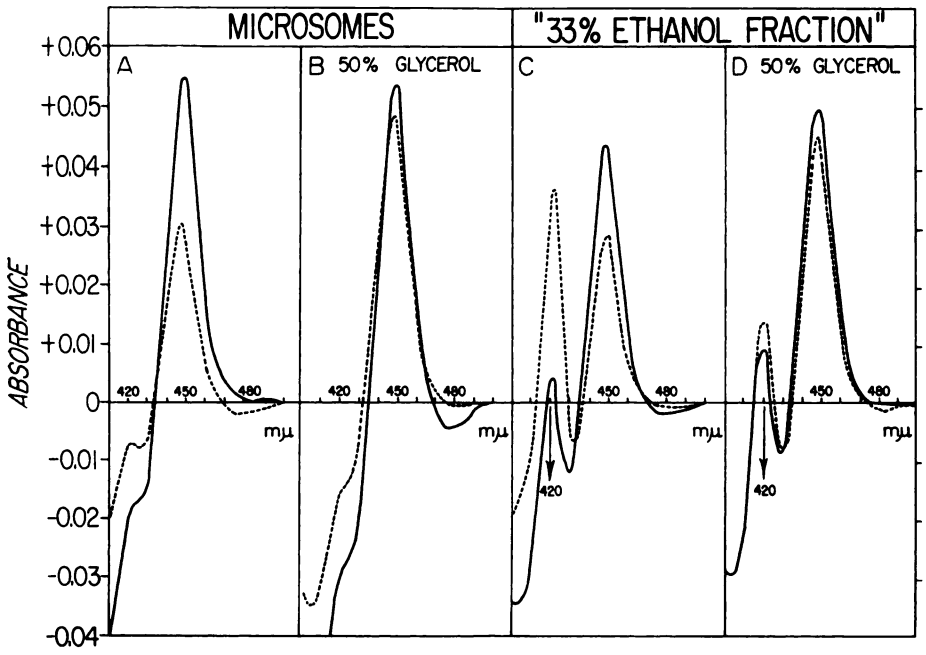

FIG. 3. *Carbon monoxide difference spectra of microsomes and "33% ethanol fraction"*

Microsomes or "33% ethanol fraction" were diluted in 100 mM potassium phosphate buffer at pH 7.4 to a concentration of 0.375 mg of protein per milliliter. Two milliliters of the mixture were placed in each of two matched Teflon-stoppered quartz cuvettes. A baseline reading was established on the Cary Model 15 recording spectrophotometer. Carbon monoxide was then bubbled through the contents of the sample cuvette for 30 sec, and 10 mg of solid $Na_2S_2O_4$ was added to both the reference and sample cuvettes. The cuvettes were stoppered and mixed by inversion; the difference spectra were then recorded. These spectra are uncorrected for small baseline variations which did not exceed ±0.002 from the absorbance at 500 mμ. (A) Difference spectra of freshly prepared microsomes (solid line) and after storage for 14 days at 4° (broken line). (B) Same as (A), except that samples were stored in 50% glycerol. (C) Difference spectra of freshly prepared "33% ethanol fraction" (solid line) and after storage for 14 days at 4° (broken line). (D) Same as (C) except that samples were stored in 50% glycerol. All samples were stored at a concentration of 7.5 mg of protein per milliliter in 25 mM potassium phosphate at pH 7.4 in the presence or absence of glycerol, and glycerol, and were diluted 20-fold with 100 mM potassium phosphate buffer at pH 7.4 just prior to recording of the spectra. Glycerol at a final concentration of 2.5% did not affect the spectra.

which had been stored in its absence did not alter either the quantity of P-450 or the benzpyrene hydroxylase activity. The direct addition of glycerol to the assay system in concentrations up to 40% had little influence on the amount of benzpyrene metabolized.

The experiments illustrated in Figs. 4–6 suggest that there is a direct correlation between the loss of P-450, produced by several methods, and the capability of these enzyme preparations to catalyze the hydroxylation of benzpyrene. Figure 4 shows the percentage of both P-450 and benzpyrene hydroxylase activity remaining after ultrasonic treatment for various periods of time. The "33% ethanol fraction" (8 mg of protein per milliliter) was subjected to ultrasonic vibrations at 0° and, after various periods of time, aliquots were removed and analyzed for their P-450 content and benzpyrene hydroxylase activity. A parallel decrease in these two components was observed with the length of time of sonic treatment.

Similar results were obtained when the "33% ethanol fraction" was subjected to treatment with snake venom phospholipase

TABLE 2

The effects of aging on benzpyrene hydroxylase activity and the protective effect of glycerol

The assay conditions are described in the section Materials and Methods. Freshly prepared microsomes and the "33% ethanol fraction" prepared therefrom were diluted with an equal volume of either buffer or glycerol, and immediately assayed for benzpyrene hydroxylase activity. The enzyme preparations were then stored for 30 days at 4° and assayed again. The incubation time was 30 min.

Enzyme preparation	Quantity of protein (mg)	Benzpyrene metabolized	
		Fresh (mμmoles)	Stored at 4° for 30 days (mμmoles)
Microsomes, no glycerol	0.5	27.5	9.8
	1.0	35.0	15.5
Microsomes in 50% glycerol	0.5	28.0	13.7
	1.0	34.4	24.0
"33% ethanol fraction," no glycerol	0.5	27.6	0.4
	1.0	40.8	1.0
"33% ethanol fraction" in 50% glycerol	0.5	29.4	7.0
	1.0	41.6	16.2

(Fig. 5). A 1% solution of *Trimeresurus flavoviridis* venom in 0.03 M Tris-chloride at pH 7.4 was heated for 5 min in a boiling water bath. The denatured proteins were removed by centrifugation, and the resulting supernatant fluid was used as the source of phospholipase. To an 18-ml portion of the "33% ethanol fraction" (8 mg of protein per milliliter), adjusted to pH 8.6 with NaOH, were added 2 ml of the heated snake venom preparation. The pH was adjusted and maintained at pH 8.6 by the

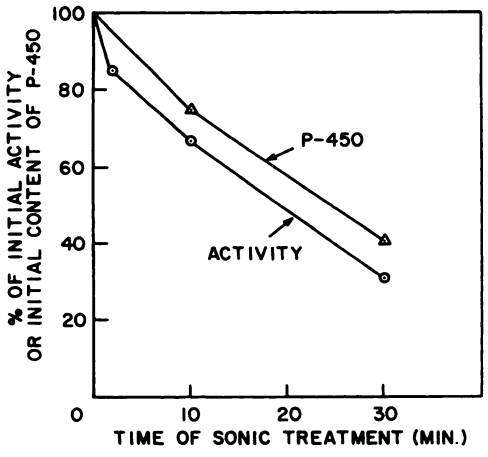

FIG. 4. *The effect of sonic oscillation on benzpyrene hydroxylase activity and P-450 content*

"33% ethanol fraction" was subjected to ultrasonic treatment on an MSE 20 KC sonic oscillator as described in the text. The content of the CO-binding pigment was determined as described in Fig. 3 with the use of the difference in the absorbancies at 450 mμ and 490 mμ as a measure of the P-450 content. Benzpyrene hydroxylase activity was assayed as described under "Materials and Methods" with 0.8 mg of protein per flask. Incubation time was 30 min.

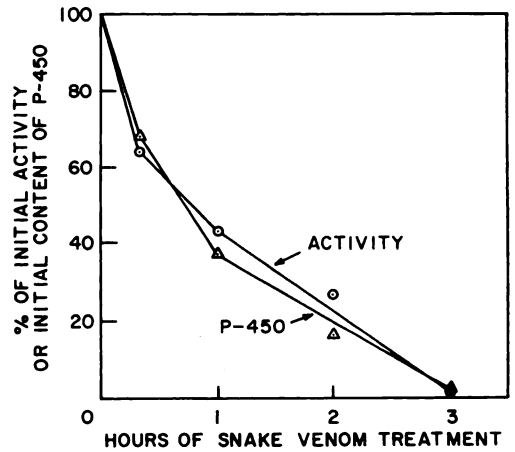

FIG. 5. *The effect of snake venom phospholipase on benzpyrene hydroxylase activity and P-450 content*

"33% ethanol fraction" was incubated with snake venom phospholipase as described in the text. The concentration of P-450 and the benzpyrene hydroxylase activities were determined as described in Fig. 4.

automatic addition of NaOH with the aid of a Radiometer automatic titrator. The reaction was carried out at 23° under pure nitrogen. After various periods of time, aliquots were removed, the pH was adjusted to 7.4 with acetic acid to terminate the action of the snake venom, and they were dialyzed overnight against 0.05 M Tris-chloride at pH 7.4. The P-450 content and benzpyrene hydroxylase activities were then determined. Again there appeared to be a direct correlation between the amounts of P-450 remaining and the quantity of benzpyrene hydroxylated.

Incubation of the enzyme at various pH values prior to assay resulted in parallel losses of P-450 and benzpyrene hydroxylase activity (Fig. 6). Two portions of "33% ethanol fraction" were used. With the temperature maintained at 0–4°, either ice-cold 0.25 N acetic acid or 0.25 N sodium hydroxide was added to the separate samples. At various pH readings, aliquots were removed and allowed to stand for 30 min. At the end of this period each aliquot was readjusted to pH 7.4 by the appropriate addition of acid or base, and the various samples were dialyzed overnight against 0.05 M Tris-chloride at pH 7.4. The samples when tested showed parallel changes in benzpyrene hydroxylase activity and P-450 content. These findings strongly suggest that the P-450 concentration limits the rate of the hydroxylation reaction under these experimental conditions.

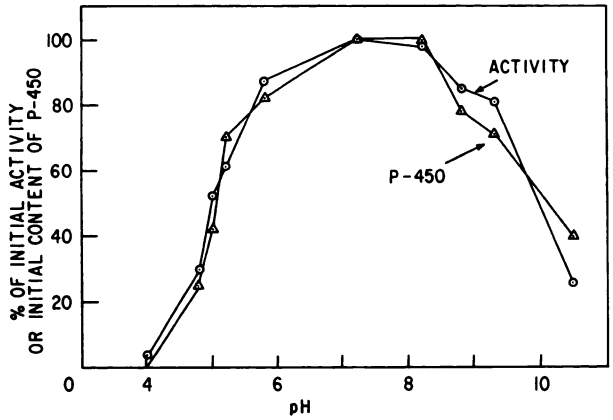

FIG. 6. *P-450 content and benzpyrene hydroxylase activity after incubation at various pH values*
"33% ethanol fraction" was incubated at various pH values as described in the text, and the P-450 content and benzpyrene hydroxylase activity were determined as described in Fig. 4.

Induction Experiments

Experiments on the induction of drug-metabolizing activity suggest that the levels of P-450 may not alone control the reaction rate under some circumstances. It is well documented (10, 11, 20) that inducers of microsomal enzymes elevate the rate of metabolism of several foreign substances to quite different extents. The results presented in Figs. 7–9 and Table 3 illustrate that for a given rise in the level of P-450, three different enzyme activities were stimulated to quite different extents. The P-450 content of microsomes derived from rats treated with either phenobarbital or benzpyrene (see Materials and Methods) is increased over that of controls, when expressed on the basis of microsomal protein content. The ratio of P-450 content present in microsomes from benzpyrene-treated, phenobarbital-treated, and control animals was 2.2:1.7:1. Hence, the two treatments did not induce markedly different elevations in the hemoprotein levels. These drug treatments also increased the rate of hydroxylation of benzpyrene by such microsomes and the "33% ethanol fractions" prepared therefrom (Fig. 8). However, benzpyrene administration was considerably more effective in increasing the hy-

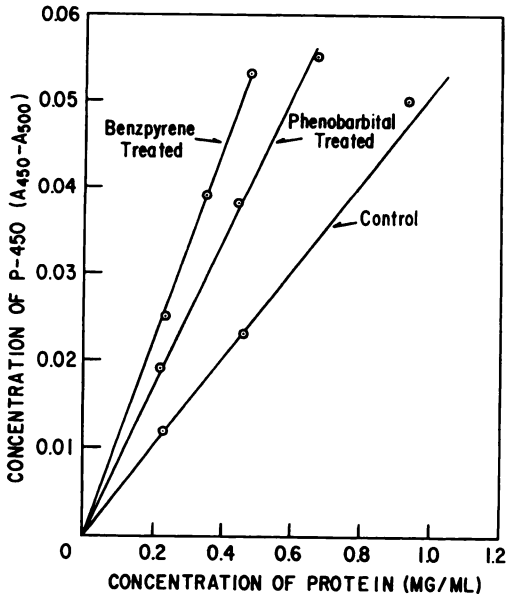

FIG. 7. *P-450 content of microsomes derived from induced and control animals*

Recently weaned male rats were treated with either phenobarbital, benzpyrene, or sesame oil as described under Materials and Methods. Microsomes were prepared from the livers of these animals and the P-450 content of the microsomes was determined as described in Fig. 4. This content was determined at three different protein concentrations for each sample as shown in the figure. The three lines represent microsomes from controls, phenobarbital-treated, and benzpyrene-treated rats, respectively.

droxylation rate than the P-450 content. The ratios of hydroxylase activities were 5.8:1.4:1 and 7.0:1.5:1 for the microsomes and Triton-treated microsomes, respectively, isolated from the animals receiving benzpyrene, phenobarbital, or sesame oil alone.

Table 3 illustrates an analogous pattern of induction for zoxazolamine hydroxylase. However, the ratios of hydroxylase activities are somewhat different, being about 5.3:2.2:1 for both the microsomes and "33% ethanol fraction" in the case of the benzpyrene-treated, phenobarbital-treated, or control animals, respectively.

Yet a third assay, shown in Fig. 9, revealed that the same enzyme samples used in the determination of benzpyrene and zoxazolamine hydroxylase, contained far greater *p*-nitroanisole-*O*-demethylase activity in the preparations from phenobarbital-treated animals than from either the carcinogen-treated or control groups. The ratios in this case were 3.5:9.6:1 for benzpyrene, phenobarital, and untreated rats, respectively. The absolute rates for all three enzymic reactions presented in Figs. 8 and 9, and in Table 4, were of the same order of magnitude. As can be seen from these figures, the rate of metabolism of benzpyrene, zoxazolamine, and *p*-nitroanisole by noninduced preparations was in the range of 1–3 mμmoles of substrate converted per milligram of protein per minute.

It is therefore evident that although the amount of P-450 is increased by prior treatment with both phenobarbital and benzpyrene, the levels of various induced enzymic activities do not necessarily correlate with the increases in the carbon monoxide-binding pigment.

Since treatment with snake venom phospholipase resulted in reduction of benzpyrene hydroxylase activity in direct proportion to the destruction of P-450 in enzyme preparations derived from benzpyrene-treated animals (Figs. 4–6), the possibility was considered that the loss of hydroxylase activity might be due solely to the destruction of this rate-limiting pigment rather than to the inactivation of other components of the system. Because microsomes from control animals contain a relatively high proportion of P-450 in relation to their benzpyrene hydroxylase activity, it was thought that perhaps the addition of these control microsomes to an induced preparation whose P-450 had been destroyed might therefore result in a reconstruction of a high degree of benzpyrene metabolism. When microsomes from benzpyrene-treated animals were subjected to overnight treatment with *Trimeresurus flavoviridis* venom at 4° as described previously, this treatment resulted in total conversion of the P-450 hemoprotein to P-420, and to a complete loss of the capacity to metabolize benzpyrene. However, the addition of either microsomes or "33% ethanol fraction" of control rats to the phospholipase-treated sample resulted in no greater

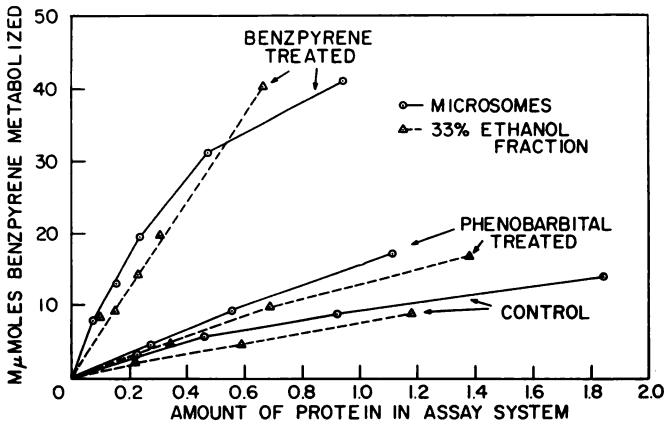

FIG. 8. *Induction of benzpyrene hydroxylase*

Benzpyrene hydroxylase activity was determined as described under Materials and Methods for the microsomes derived from induced and noninduced animals used in the experiment of Fig. 7 and also for the "33% ethanol fraction" prepared from these microsomes. Experiments with intact microsomes are represented by solid lines and those utilizing the Triton-treated material are indicated by broken lines. Protein concentrations are shown on the abcissa. Incubation time was 20 min.

rate of hydroxylation than was obtained with the control preparations alone. It may be concluded that either the snake venom treatment destroys other essential components of the enzyme system in addition to altering P-450, or alternatively the P-450 of the control preparations cannot exert its essential function for another enzymic system because it is bound in such a way as to be unavailable to the enzymes in the snake venom-treated preparation of in-

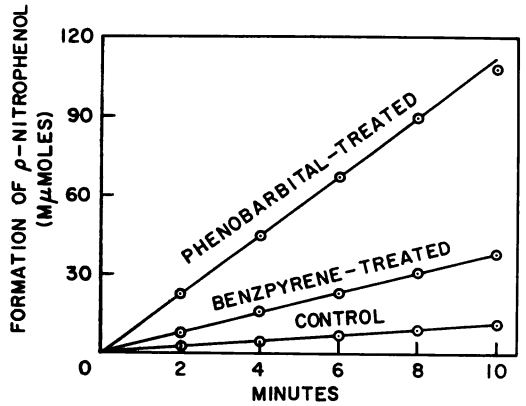

FIG. 9. *Induction of p-nitroanisole-O-demethylase*

The determination of *p*-nitroanisole-*O*-demethylase activity has been described under Materials and Methods. The rates represented are those for "33% ethanol fraction" from control, phenobarbital, and benzpyrene-treated rats. The quantity of the enzyme preparations added was equivalent to 1.8 mg of protein. As described by Netter (27, 28), an increase in absorbancy at 420 mμ of 0.001 in a 1.0-cm light path cuvette was assumed to be equivalent to the production of 0.299 mμmole per millimeter of *p*-nitrophenol.

TABLE 3

Induction of zoxazolamine hydroxylase activity in rat liver by phenobarbital and benzpyrene administration

Recently weaned male rats were treated with either phenobarbital, benzpyrene, or sesame oil as described under Materials and Methods. Microsomes were prepared from the livers of these animals, and the "33% ethanol fraction" was obtained from the microsomal fraction. Benzpyrene hydroxylase activity was assayed as described under Materials and Methods. The incubation time was 30 min.

Treatment of animals	Amount of zoxazolamine metabolized	
	Microsomes (mμmoles per mg of protein)	"33% Ethanol fraction" (mμmoles per mg of protein)
Control	11.0	11.5
Phenobarbital treatment	24.0	26.2
Benzpyrene treatment	58.0	59.5

duced microsomes. Likewise, P-450 prepared from bovine adrenal mitochondria by sonic disintegration as described by Omura *et al.* (22) failed to reactivate the benzpyrene hydroxylase activity of the snake venom treated microsomes.

Ernster, Orrenius and co-workers (18, 32, 33) have shown that the changes in the activity of oxidative demethylation of aminopyrine closely parallel the changes in P-450 content of microsomes obtained from animals treated with phenobarbital. This is true both during the induction phase following the administration of the barbiturate and the regression phase after cessation of drug administration. Similarly, as seen from the work of Netter (27, 28), and as confirmed in Fig. 9, phenobarbital administration produces a very large stimulation of *p*-nitroanisole-*O*-demethylase activity although the relative increase in P-450 content induced by this treatment was considerably lower in this case.

It would appear from the results of the experiments illustrated by Figs. 4–6 that the level of P-450 may be rate limiting in microsomal hydroxylations, under the specific conditions when a relatively high level of drug metabolism has been produced by induction. It should be remembered that TPNH-dependent microsomal hydroxylations are complex processes involving not only TPNH, oxygen, carbon monoxide-binding pigment, and the various specific (or nonspecific) drug-metabolizing enzymes, but perhaps also nonheme iron and flavoproteins as well (22, 32, 34).

Flavin Requirement

Because P-450 is extremely labile at low pH (cf. Fig. 6), it was difficult to establish a flavin requirement for the hydroxylation of benzpyrene. However, methods patterned after those of Pettit *et al.* (34) were designed to dissociate flavins from the enzyme preparation by gel filtration on Sephadex

TABLE 4

Stimulation of acid-treated benzpyrene hydroxylase preparations by flavins

The Sephadex-fractionated "33% ethanol fraction" was prepared by the addition of solid KCl to the "33% ethanol fraction" at 0° to a final concentration of 3.0 M. The preparation was stirred for 90 min, and the pH was then adjusted to 5.2 by the careful addition of 0.5 N acetic acid. The sample was incubated at 0° for 60 min, and the pH was then raised to 5.5 with 0.5 N KOH. A small precipitate was removed by centrifugation at 20,000 *g* for 10 min. From 3 to 5 ml of the supernatant fluid was introduced onto a column of Sephadex G-25 previously equilibrated against 3.0 M KCl, 0.01 M Tris-acetate buffer at pH 5.5. The column measured 30 × 1.8 cm; it had a bed volume of 175 ml and a void volume of 50 ml. The protein was eluted with 3 M KCl, 0.01 M Tris-acetate at pH 5.5. Fractions were collected, and their absorbancies at 280 mμ were determined. The 3 or 4 most concentrated protein fractions of the single peak were combined and dialyzed for 3 hr against 3 changes of 0.05 M Tris-chloride buffer at pH 7.4. The fraction was then assayed for benzpyrene hydroxylase activity as described under Materials and Methods, both in the absence and the presence of the flavins indicated in the table. The amount of enzyme fraction used in each assay was equivalent to 0.4 mg of protein. The incubation time was 30 min.

Enzyme preparation	Flavin added	Benzpyrene metabolized (mμmoles)
1. Sephadex fractionated "33% ethanol fraction"	None	2.8
	FAD, 2 μM	7.2
	FAD, 200 μM	9.0
	FMN, 2 μM	8.6
	FMN, 200 μM	9.0
	FAD, 2μM + FMN, 2 μM	8.5
	Riboflavin, 2 μM	2.9
	Riboflavin, 200 μM	2.8
2. Untreated "33% ethanol fraction"	None	15.9
	FAD, 200 μM	17.2
	FMN, 200 μM	16.5

C-25 following incubation of the "33% ethanol fraction" at high salt concentrations and at pH 5.2. Preparations treated in this way exhibited a markedly decreased hydroxylase activity which was stimulated from 25 to 300% by the addition of low concentrations of either FAD or FMN (but not by riboflavin) (Table 4). The hydroxylations catalyzed by untreated "33% ethanol fraction" or by microsomes were unaffected by either of these compounds.

However, notwithstanding the participation of flavins or other factors, it seems reasonable to suppose that the major barrier to the solubilization of hepatic microsomal TPNH-dependent hydroxylation system is the lability of the essential pigment, P-450, which is converted to an inactive form following treatments designed to solubilize the microsomes. The observation that the presence of glycerol tends to protect both against inactivation of P-450 and loss of hydroxylase activity suggests that the conversion process may be hydrophobic in nature, and that solubilization attempts performed in media of a dielectric constant different from that of water might perhaps yield soluble and active hydroxylase preparations.

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