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THE INDUCTION OF BENZPYRENE HYDROXYLASE IN THE ISOLATED PERFUSED RAT LIVER*

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Abstract—Induction of microsomal benzpyrene hydroxylase apparently occurs in the isolated perfused rat liver. This was demonstrated by assays of the benzpyrene hydroxylase activities of liver perfused with 3, 4-benzpyrene. These were compared with the enzyme activities in simultaneously perfused control livers and nonperfused livers from animals killed both at the time of mounting of the perfused livers and at the time of their removal for assay. The maximal enzyme induction was obtained after 6·5 hr of perfusion. The degree of stimulation at this time (two to threefold) was comparable to that obtainable *in vivo* for the same period, indicating that induction in the intact animal may occur independently of extrahepatic factors. Actinomycin D inhibited the increase in benzpyrene hydroxylase activity in perfused livers, indicating that an actual synthesis of new enzyme probably occurs. Microsomal zoxazolamine hydroxylase activity in perfused livers compared favorably with that observed *in vivo*, but addition of 3, 4-benzpyrene to the perfusate did not significantly affect this metabolic pathway during the intervals studied. Even *in vivo*, 3, 4-benzpyrene activation of zoxazolamine hydroxylase is apparently slow, being detected at 12 hr but not 6·5 hr after treatment with 3, 4-benzpyrene. Studies of enzyme activity after death revealed a rapid decline of zoxazolamine hydroxylation, so that maintenance of this enzymic activity appears to be a good criterion of the viability of microsomal drug-metabolizing enzymes in perfused livers. The methods employed appear to be useful for the further study of rapidly acting microsomal enzyme stimulators.

FALK *et al.*[1] have shown that the polycyclic aromatic hydrocarbon 3,4-benzpyrene is metabolized largely by hydroxylation and conjugation in the rat liver. Conney *et al.*[2] demonstrated that hepatic benzpyrene hydroxylation is catalyzed by a microsomal enzyme system in the normal, untreated rat. The activity of this enzyme system is significantly enhanced in the rat within 3 hr after i.p. injection of the rat with its substrate, 3,4-benzpyrene; several other metabolic pathways are stimulated 18 hr after such pretreatment. Gillette's group[3, 4] report that ACTH and anabolic steroids can also affect hepatic microsomal drug metabolism, but longer pretreatment periods (three days and four weeks respectively) are necessary to obtain stimulation. Gillette[4] also suggests that ACTH and benzpyrene stimulate hepatic microsomal zoxazolamine hydroxylation in an additive manner and therefore apparently by different mechanisms.

It is proposed that the inducing action of 3,4-benzpyrene on hepatic microsomal benzpyrene hydroxylase activity may be by a direct effect upon the liver and would

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not depend upon any concomitant or previous effect of 3,4-benzpyrene on other functioning organs or tissues of the body. We decided to test this hypothesis by using the isolated rat liver preparation. Price and Dietrich[5] have previously employed this technique to study the induction of tryptophan peroxidase by its normal substrate, and Sayre *et al.*[6] have studied the induction of threonine dehydrase by this method.

METHODS

Perfusion techniques. Livers were obtained from unfasted, male Long-Evans rats, weighing 120–150 g, which were maintained on regular Purina lab chow and water *ad libitum* until immediately preceding each experiment. The perfusion fluid used was a mixture of two thirds Weymouth's solution[7] and fraction V bovine serum albumin (35·2 mg/ml Weymouth's solution) and one third whole blood obtained from unfasted, adult (300-350 g) Holtzman rats which were likewise maintained on regular Purina lab chow and water *ad libitum* until the experiment. The blood-donor rats were lightly anesthetized with ether and the blood taken from the abdominal aorta. The blood used in the perfusion fluid contained approximately 25 units/ml heparin. The perfusion fluid also contained 60 μg streptomycin/ml, and 35 μg penicillin G potassium/ml.

Two perfusion apparatuses were set up in the same constant-temperature cabinet, such that control livers could be perfused simultaneously with test livers. These were mounted by essentially the technique of Plaa and Hine.[8] Temperatures within the perfusion cabinet were maintained within the range 36·0°-38·5°; pH readings were taken hourly and maintained within the range 7·20-7·40 (Beckman pH meter) by appropriate additions of 0·55 M $NaHCO_3$. Oxygen and carbon dioxide tensions of the perfusion medium were maintained at 250–350 mm Hg and 25–40 mm Hg, respectivly, by aeration with a 95% – 5% mixture of oxygen–carbon dioxide.Perfusion pressures at the hilus of the livers were maintained between 185 and 220 mm perfusate. To obtain livers for perfusion, the Long-Evans rats were lightly anesthetized with ether and the spinal cord sectioned at the level of C-6 so that further manipulation could be carried out without requirement of continuous anesthesia. The portal vein was cannulated, and the liver excised and mounted on a nylon mesh filter which allowed the free flow of perfusate through the liver. The duration of ischemia (time from occlusion of the portal vein until resumption of circulation) was less than 6 min (usually about 4 min).

The amount of perfusate solution contained in each perfusion apparatus was approximately 170 ml. For perfusions lasting less than 12 hr, 40 ml of perfusate was exchanged at 4 and 7 hr without interrupting the perfusion flow. For 12-hr perfusions the perfusate was totally exchanged at 6 hr. Six mg 3,4-benzpyrene/ml was partially dissolved and suspended in a separate volume of perfusate solution; a total of 6 mg of 3,4-benzpyrene was added initially to the fluid perfusing the liver and 3 mg every 30 min thereafter during perfusion. In experiments involving actinomycin D, 2 mg/ml was dissolved in a separate volume of perfusate solution, and 1 ml of this solution was added to the fluid perfusing the liver at the onset of perfusion; 1 ml was also added upon exchange of perfusate.

The microsomal enzyme activities of perfused test livers were compared (paired "t" test) to the activities of their respective control perfused livers. In addition, the activities of the perfused test livers were compared to those of unperfused livers

obtained (1) from animals killed at the termination of perfusion (control A), and (2) from animals killed shortly before commencement of perfusion (control B).

Factors that might affect drug metabolism in the perfused liver

The effects of penicillin, streptomycin, heparin and actinomycin D, added *in vitro* as well as injected *in vivo*, and sham operations on animals both with the stimulated and nonstimulated metabolism of 3,4-benzpyrene and of zoxazolamine were determined. For *in vivo* experiments, 2 and 4 mg heparin/kg, 40 mg penicillin G potassium/kg, 68 mg streptomycin/kg, and 2 mg actinomycin D/kg were injected into both benzpyrene-treated (20 mg/kg, i.p., in corn oil) and corn oil-treated, weanling Long-Evans rats 12 hr before sacrifice and assay and compared to suitable controls. For *in vitro* experiments 0·5, 1·0 and 2·0 mg heparin, 0·25, 0·5 and 1·0 mg penicillin G potassium, 0·4, 0·8 and 1·6 mg streptomycin, and 0·04, 0·08 and 0·16 mg actinomycin D per incubate (5 ml total volume) were added in order to determine the effects of these agents on the hepatic microsomal metabolism of 3,4-benzpyrene and zoxazolamine. Each incubate contained an aliquot of the pooled 9,000-*g* supernatant from three livers and was assayed in triplicate. The results were expressed as the mean of the triplicate assay. The sham operations were the same as for perfusing the livers except that the portal vein was not cannulated. The animals were allowed 1 hr to recover from ether anesthesia.

In addition, *in vivo* stimulation of benzpyrene and zoxazolamine hydroxylase activities were carried out in order to compare results with those obtained in the isolated perfused livers. Male Long-Evans rats (120–150 g) were employed and were injected i.p. with 20 mg 3,4-benzpyrene in corn oil per kg, 3, 4, 6·5, and 12 hr prior to assay for benzpyrene and zoxazolamine hydroxylase activities. Group comparisons were made by Student's "t" test.

Post-mortem studies

The normal post-mortem decline of hepatic microsomal benzpyrene and zoxazolamine hydroxylase activities was determined in order to compare it with that observed in the perfusion experiments. Groups of male Long-Evans rats (120–150 g) were employed, half of which were injected i.p. 12 hr before sacrifice with 20mg/3,4-benzpyrene kg; the other half remained uninjected. The animals were killed by cervical dislocation and kept at room temperature with livers left *in situ* for various time intervals. Hepatectomies were then performed and determinations of benzpyrene and zoxazolamine hydroxylase activities were made on livers from three benzpyrene-treated and three untreated animals at each time interval.

Assay procedures

Benzpyrene and zoxazolamine hydroxylase activities were assayed by modifications of the methods of Conney *et al.*[2, 9] Both perfused and unperfused livers were placed immediately on ice, blotted, weighed, and ground with a Potter-Elvehjem homogenizer (Teflon pestle) in two volumes of ice-cold 1·15% KCl solution. This homogenate was then centrifuged at 1°–3° (International portable refrigerated centrifuge, model PR-2) at 9,000 *g* for 30 min.

Benzpyrene assay. One ml of hepatic 9000-*g* supernatant solution was incubated for 30 min in a Dubnoff shaking incubator under oxygen with 2·5 ml phosphate buffer

(0·1 M, pH 7·34), 1·0 ml phosphate buffer solution containing glucose-6-phosphate (10 mg/ml), and 0·3 ml of a benzpyrene solution (0·5 mg/ml in acetone). The reaction was stopped by adding 1·0 ml of cold ethanolic KOH (158 ml H_2O, 792 ml 95% ethanol, and 198 *g* KOH). A sufficient quantity of ethanolic KOH was subsequently added to bring the total volume to 15 ml. The sample was then stored in the cold for 24 to 48 hr, after which 0·3 ml of the mixture was shaken for 20 min. with 3 ml of 11 N KOH and 10 ml Skellysolve B (petroleum ether) and centrifuged for 5 min. Fluorescence of the Skellysolve B layer was determined by a Turner (model 110) photofluorometer with primary filter, No. 110–811, which transmits maximally at 360 mμ, and secondary filter No. 110–816, which transmits wavelengths longer than 415 mμ. The amount of 3,4-benzpyrene metabolized was calculated as the difference between the amounts extracted from the zero time flasks (unincubated) and the incubated flasks. The fluorescence was linear in the range assayed (0 to 1·0 μg/10 ml petroleum ether). Little or no fluorescent material was extracted from the flasks which contained only tissue of livers untreated with benzpyrene. In the isolated, perfused, benzpyrene-treated livers, the amounts of residual benzpyrene remaining in the tissue owing to the perfusion were determined, and appropriate corrections were made.

Zoxazolamine assay. One ml of the hepatic 9,000-*g* supernatant solution was incubated for 60 min in a Dubnoff shaking incubator under oxygen with 2·3 ml phosphate buffer (0·1 M, pH 7·34); 1·0 ml phosphate buffer solution containing glucose-6-phosphate (10 mg/ml), nicotinamide (30·5 mg/ml), and magnesium sulfate (6·2 mg/ml); 0·2 ml NADP (2·0 mg/ml in phosphate buffer); and 0·5 ml zoxazolamine (1·0 mg/ml in phosphate buffer; the drug was initially dissolved in a small volume of 0·1 N HCl and then made up to final concentration with the buffer). After incubation, 2·0 ml of the incubate was shaken for 30 min with 25 ml heptane containing 1·5% isoamyl alcohol and 0·5 ml of 1·0 N NaOH and centrifuged for 5 min. After aspiration of the aqueous layer, 7·0 ml of 0·1 M acetate buffer (pH 5·6) was shaken for 5 min with the heptane layer and centrifuged for 5 min. Fifteen ml of the heptane layer was then shaken with 7·0 ml 0·1 N HCl and centrifuged for 5 min. The optical density of the aqueous phase was read at 278 mμ against a 0·1 N HCl blank on a Beckman DU spectrophotometer. The amount of zoxazolamine metabolized was calculated as the difference in the amounts extracted from the zero-time flasks (unincubated) and the incubated flasks. Results for both benzpyrene and zoxazolamine assays were expressed as micromoles substrate metabolized per gram nitrogen per incubation time.

Nitrogen determinations were made by boiling 0·9 ml distilled water, 0·1 ml of the 9,000-*g* supernatant solution, and 1·0 ml 50% H_2SO_4 solution with a Hengar selenium granule as catalyst for 30 min. After cooling slightly, one drop of 30% H_2O_2 was added and the solution boiled again for 5–10 min. The solution was again cooled and the volume adjusted to 50 ml with distilled water. This was mixed and centrifuged for 15 min to sediment the selenium. One ml (in duplicate) of the supernatant was mixed with 5·0 ml freshly prepared Nessler's reagent (1·0 ml Nessler's stock solution from E. H. Sargent & Co. plus 5·0 ml 1·0 N NaOH) and read at 420 mμ on a Coleman Jr. spectrophotometer with NH_4Cl as a standard.

The data from all experiments were analyzed statistically by methods described in Snedecor.[10] The level of significance chosen was $P < 0.05$.

RESULTS

Effects of injected 3,4-benzpyrene, actinomycin D, heparin, streptomycin, and penicillin on hepatic microsomal drug metabolism in rats

In vivo, the minimum time necessary significantly to enhance hepatic microsomal benzpyrene hydroxylation with injected 3,4-benzpyrene was 6·5 hr, whereas significant enhancement of zoxazolamine hydroxylation was not obtained until 12 hr (Table 1). In animals not treated with benzpyrene, pretreatment with actinomycin D did not

TABLE 1. TIME COURSE OF INDUCTION OF BENZPYRENE HYDROXYLASE AND ZOXAZOLAMINE HYDROXYLASE *in vivo*

Elapsed time* (hr)	Metabolism+ of 3, 4-benzpyrene			Metabolism+ of zoxazolamine		
	Benzpyrene treated	Control	Ratio‡	Benzpyrene treated	Control	Ratio‡
2	21·1 ± 3·8	19·0 ± 5·0	1·11	130 ± 13	107 ± 51	1·21
4	28·3 ± 7·7	22·1 ± 3·8	1·28	137 ± 15	112 ± 20	1·22
6·5	46·1 ± 3·8	21·1 ± 6·8	2·19§	145 ± 17	118 ± 13	1·22
12	43·9 ± 7·5	17·5 ± 6·6	2·51§	140 ± 24	94 ± 16	1·49§

* Time from injection of animals (20 mg/kg i.p. in corn oil) to removal of livers for assay.
+ Expressed as μmoles substrate metabolized per g nitrogen per time of incubation (see Methods). All groups had 6 animals.
‡ Ratio of metabolism of benzpyrene-treated to control anumals.
§ Benzpyrene-treated significantly different from control ($P < 0·05$).

TABLE 2. EFFECTS OF VARIOUS *in-vivo* TREATMENTS ON THE HEPATIC MICROSOMAL METABOLISM OF 3,4-BENZPYRENE AND ZOXAZOLAMINE IN RATS

Expt.	Treatment of test animals*	3, 4-Benzpyrene metabolism†			Zoxazolamine metabolism†		
		Control	Test	Test‡ / control	Control	Test	Test‡ / control
	Compared to Non-treated control animals						
I	3, 4-Benzpyrene	17·5 ± 6·6	43·9 ± 7·5	2·51§	94 ± 16	140 ± 24	1·49§
II	Penicillin + Streptomycin + heparin	12·7 ± 4·1	13·2 ± 4·7	1·04	80 ± 16	76 ± 12	0·94
III	Actinomycin D	19·1 ± 5·9	18·5 ± 6·7	0·97	135 ± 29	131 ± 28	0·97
IV	Sham operation	15·3 ± 5·2	14·1 ± 3·4	0·92	104 ± 30	98 ± 17	0·94
	Compared to 3, 4-benzpyrene-treated control animals						
V	Penicillin + streptomycin + heparin + 3, 4-benzpyrene	60·2 ± 5·2	62·3 ± 6·0	0·97	280 ± 33	246 ± 47	0·88
VI	Actinomycin D + 3, 4-benzpyrene	44·4 ± 1·6	26·9 ± 1·6	0·61§	181 ± 24	110 ± 22	0·61§
VII	Sham operation + 3, 4-benzpyrene	32·4 ± 4·7	40·3 ± 6·0	1·24	217 ± 22	227 ± 33	1·05

* All agents injected i.p. 12 hr prior to sacrifice (see Methods for dosages).
† Expressed as μmoles substrate metabolized per g nitrogen per incubation time (see Methods). All groups contained at least 5 animals; all values, mean ± S.D.
‡ Ratio of the mean metabolisms of test animals to their respective controls for the individual experiments.
§ Metabolism of test groups significantly differs from that of the control group ($P < 0·05$).

significantly lower the hepatic microsomal metabolism of 3,4-benzpyrene or zoxazolamine after 12 hr. Actinomycin D did significantly ($P < 0.05$) diminish the enhancement of benzpyrene and zoxazolamine hydroxylation typically elicited by injected 3,4-benzpyrene (20 mg/kg; Table 2). Similar results for actinomycin were obtained by Gelboin and Blackburn,[11] who used 3-methylcholanthrene as the inducing agent and rats weighing 40–50 g. Heparin, streptomycin, and penicillin injected *in vivo* at the dosage levels shown in the table did not significantly ($P > 0.05$) affect the basal level of hepatic microsomal hydroxylation of benzpyrene or zoxazolamine, nor did these agents restrict benzpyrene stimulation of these metabolic pathways (Table 2). Inclusion of heparin, streptomycin, penicillin, and actinomycin D in the incubation mixtures had no effect on the metabolism of microsomes from livers of either control or benzpyrene-treated rats.

Microsomal 3,4-benzpyrene metabolism of perfused livers

Data are given of both individual experimental values (Tables 3 and 4) and means of all experiments (Fig. 1 and 2). We believe the best statistical comparison is of

TABLE 3. COMPARISONS WITH VARIOUS CONTROLS OF 3,4-BENZPYRENE AND ZOXAZOLAMINE MICROSOMAL METABOLISM IN ISOLATED PERFUSED LIVER

Perfusion time (hrs)	Metabolism* of 3, 4-benzpyrene				Metabolism* of zoxazolamine			
	Benzpyrene perfused	Control-perfused	Unperfused control A	Unperfused control B	Benzpyrene perfused	Control perfused	Unperfused control A	Unperfused control B
12	†	23·5	17·7			50	110	
12	24·9	16·8	30·2		57	81	106	
12	30·7	14·1	32·0					
12		9·4		16·6 ± 5·9	104	71	86	102 ± 16
8	26·9	19·2	13·5		129	136	117	
8		12·2	19·0	12·7 ± 4·1	94	69	98	81 ± 16
6·5	27·3		12·9		46		67	
6·5	37·5	15·4	9·0	32·1 ± 4·5	88	72	47	107 ± 13
6·5	20·9	12·6	16·6	18·6 ± 4·6	85	58	62	116 ± 18
6·5	38·5	14·5	15·9	11·3 ± 8·9	113	134	106	98 ± 19
4	24·5	16·9	15·7	9·0 ± 5·3	57	66	78	96 ± 22
4					73		47	76 ± 18
4	17·6	11·5	8·1	19·8 ± 5·9	120	78	59	96 ± 25
4	24·7	26·6	17·1	19·5 ± 7·1	45	66	32	146 ± 20
2	19·4	13·8	13·7	19·9 ± 4·5	52	127	104	99 ± 31

* Metabolism expressed as μmoles substrate metabolized per g nitrogen per time of incubation (see Methods). Both assays were performed on each benzpyrene-perfused, control-perfused, and nonperfused control A liver, and on five nonperfused control B livers. For the latter group values represent mean metabolism with standard deviations. All values in each line of the table represent data obtained from a single experiment.

† Blanks indicate that no determination was made.

perfused and unperfused livers run at the same time (paired comparisons), whereas the overall pattern is best expressed as the mean (Fig. 1 and 2).

Microsomes from livers perfused with 3,4-benzpyrene metabolized significantly ($P < 0.05$) greater amounts of benzpyrene than their respective paired control perfused livers after 6·5 and 12 hrs of perfusion (Table 3 and Fig. 2). In contrast, the benzpyrene

metabolism of benzpyrene-perfused livers differed significantly ($P < 0{\cdot}05$) from that of the respective unperfused control B livers after 6·5-hr but not after 12-hr perfusion (Table 3 and Fig. 2). When benzpyrene was added to the perfusate of both pairs of perfused livers, but actinomycin D to only one of these perfusates, high benzpyrene hydroxylase activity in the livers not exposed to actinomycin D contrasted with significantly lower activity in the livers treated with both 3,4-benzpyrene and actinomycin D (Table 4). Comparison of perfused livers treated with only actinomycin D to untreated

TABLE 4. EFFECT OF ACTINOMYCIN D ON METABOLISM IN THE ISOLATED PERFUSED LIVER*

	Metabolism‡ of 3, 4-benzpyrene				Metabolism‡ of zoxazolamine			
Expt.†	Control-perfused	Actinomycin-perfused	Unperfused control A	Unperfused control B	Control-perfused	Actinomycin-perfused	Unperfused control A	Unperfused control B
I	23·1	17·5	12·7	21·1 ± 6·8	88	55	108	118 ± 13
II	19·5	9·3	14·6	17·5 ± 6·6	48	84	96	94 ± 17
III					108	85	103	60 ± 8
IV	27·3	6·5	17·0	19·0 ± 5·0	123	112	110	107 ± 51
V	14·7	14·9	20·8	15·9 ± 4·1	85	88	84	92 ± 17
VI	14·1	14·2	13·2	15·3 ± 5·2	87	83	60	104 ± 30

* Duration of all perfusions was 6·5 hr.

† In experiments I–IV the perfusates of both control perfused- and actinomycin- perfused livers contained 3, 4-benzpyrene; in experiments V and VI no 3, 4-benzpyrene was added to either perfusate.

‡ Expressed as μmoles substrate metabolized per g nitrogen per incubation time. All values in the table represent results of single experiments except where noted by standard deviations.

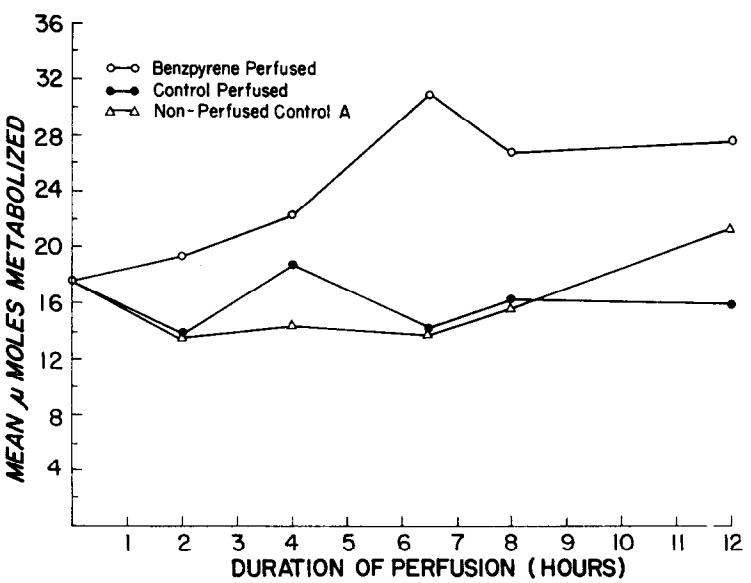

FIG. 1. Mean micromoles 3, 4-benzpyrene metabolized by all livers: benzpyrene-perfused, perfused controls, and unperfused controls A. Assays were performed at termination of perfusion and expressed as micromoles metabolized per gram nitrogen per 30 min. The value at zero time is the mean for the livers of the 45 control B animals (17·6 ± 4·8, S.D.).

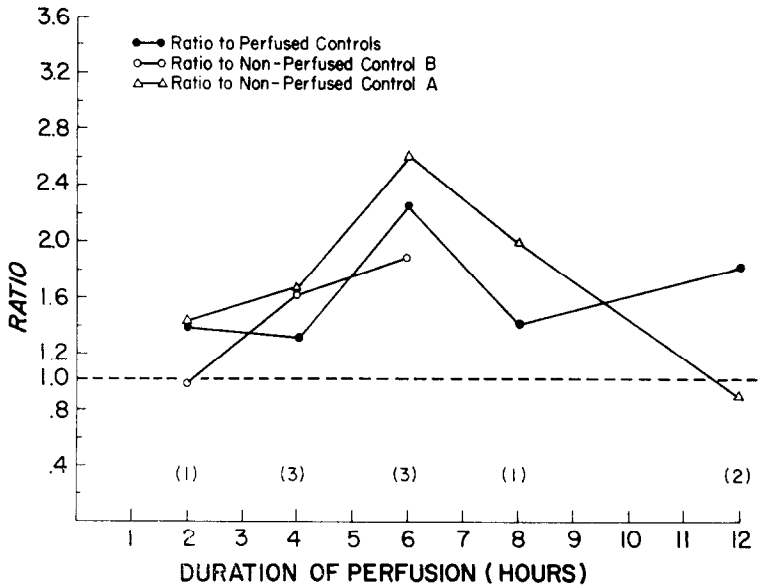

FIG. 2. Comparison of benzpyrene metabolism in benzpyrene-perfused livers to that in various control livers. Each point represents the mean paired-ratio obtained at each time interval, and numbers in parentheses represent the number of paired perfusions.

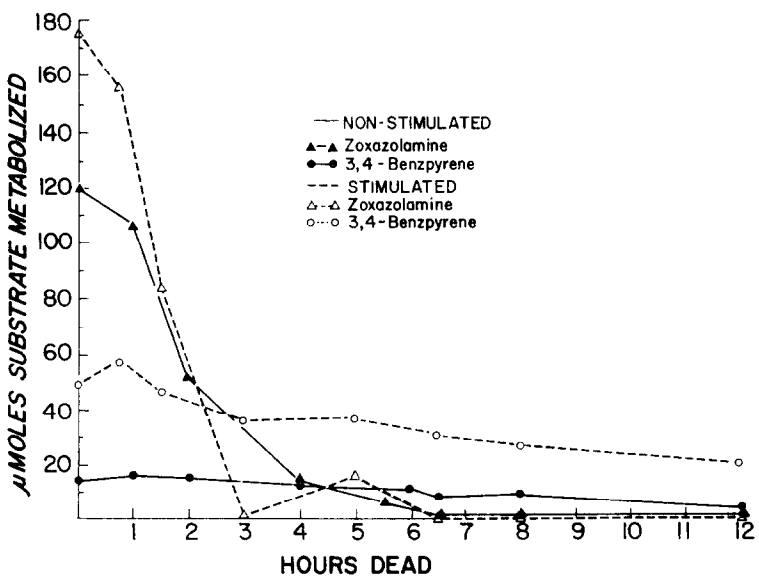

FIG. 3. Microsomal metabolism of livers assayed at varying time intervals after death. Results are expressed as mean micromoles substrate metabolized per gram nitrogen per incubation time.

perfused control livers shows that actinomycin D has no effect on basal levels of benzpyrene hydroxylase at the dose employed in our experiments.

Microsomal zoxazolamine metabolism of perfused livers

At no interval of perfusion was microsomal zoxazolamine hydroxylation of the benzpyrene-perfused livers significantly different from either perfused or unperfused control livers (Table 3). Likewise, actinomycin D had no significant effect on zoxazolamine hydroxylase activity of perfused livers, either with or without concomitant inclusion of benzpyrene in the perfusate (Table 4).

Post-mortem hepatic microsomal metabolism of benzpyrene and zoxazolamine

Post-mortem hepatic microsomal benzpyrene hydroxylase activity of both benzpyrene treated and control rats declined gradually with time (Fig. 3). Significant benzpyrene hydroxylase persisted for 12 hr in benzpyrene-treated rats and for 8 hr in untreated rats. In contrast, microsomal hydroxylation of zoxazolamine in both benzpyrene-stimulated and control rats declined over 50% within 2 hr post-mortem and was essentially absent 6·5 hr after death.

DISCUSSION

Several investigators[2, 9, 11–14] have studied the effects of the administration *in vivo* of 3,4-benzpyrene on liver microsomal enzymes and have demonstrated the capacity of this agent to stimulate certain hepatic microsomal enzyme pathways. Until the present time, however, demonstration of such stimulation has been performed only on living intact animals. Whether other concomitantly or previously functioning organs, tissues, or glands are necessary to produce this effect has heretofore been investigated exclusively by ablation experiments.

Our data demonstrate that induction of the enzyme benzpyrene hydroxylase may occur in the isolated perfused liver preparation. It should be noted that the degree of stimulation at 6·5 hr (the peak stimulation obtained in our experiments) was comparable with that of the *in vivo* stimulation. Also, the ability of the microsomes of isolated perfused control livers to metabolize 3,4-benzpyrene and zoxazolamine compared favorably with that of unperfused control livers, regardless of the duration of the perfusion. The data obtained in studies on enzyme activity after death, on the other hand, demonstrate a very rapid decline of zoxazolamine hydroxylase activity and a slow decline of benzpyrene hydroxylase activity with time. Thus, zoxazolamine hydroxylase activity may be regarded as a good criterion of microsomal viability in each experiment. Inability significantly to enhance zoxazolamine hydroxylase activity by perfusing livers with benzpyrene may reflect involvement of extrahepatic influences or a very slow induction process. Even *in vivo*, stimulation of zoxazolamine metabolism could be demonstrated only at 12 hr after benzpyrene injection, not at 6·5 hr.

The stimulated benzpyrene hydroxylase activity may not be as well maintained in the isolated perfused liver as in the intact animal. This may be indicated by the rapid "leveling-off" of activity after 6·5 hr of perfusion. Such a leveling off may be due to the accumulation of metabolites or other breakdown products in the perfusate, competitive inhibition of excess substrate in the perfusate, or increased lability of the stimulated enzyme. The data obtained from post-mortem studies tend to indicate that increased lability is not a major factor.

For 12-hr perfusions, the low ratio of activity of livers perfused with benzpyrene to unperfused (control B) livers may be due to what appears to be a *circadian* variation in the basal level of metabolism, i.e. a relatively high activity of microsomes from animals killed at 9:00 a.m. These findings are supported by the recent studies of Ertel *et al.*[15]

Studies with actinomycin D, which inhibits DNA-directed RNA synthesis[16] and thus the synthesis of new enzymes, support the hypothesis that the induction of new enzyme can occur in the isolated perfused liver and that the observed enhancement of activity as shown by the data was due to an actual induction of the synthesis of new enzyme.

The results indicate that the mechanism of induction probably involves a direct action of 3,4-benzpyrene on the liver. Whether factors contained in the perfusate are necessary for enzyme induction by benzpyrene hydroxylase cannot be completely ruled out, but the following points minimize this: (1) blood for the perfusate was taken from larger animals which, in our experience, are not stimulated by pretreatment with 3,4-benzpyrene at periods of 12 hr or less; (2) the blood used for the perfusate was diluted by a factor of one third, yet the response of perfused livers very closely simulated the *in vivo* situation; (3) most hormones that may be present in the perfusate are rapidly metabolized by the liver.

The methods used appear to be suited to further studies of this problem.

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