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REGULATION OF RAT LIVER EPOXIDE HYDRATASE ACTIVITY BY PHENOBARBITAL AND 3-METHYLCHOLANTHRENE

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Abstract—Phenobarbital-pretreatment of rats increased liver microsomal epoxide hydratase activity 2.6-fold over controls even after elimination of inherent latency problems. However, 3-methylcholanthrene pretreatment of rats does not alter the levels of hepatic epoxide hydratase activity. Epoxide hydratase was purified from control rats or rats pretreated with phenobarbital or 3-methylcholanthrene. The enzymes isolated from all three sources appear to be very similar in size, immunological activity and specific activity. These experiments strongly suggest that phenobarbital stimulates epoxide hydratase activity by selectively increasing microsomal content of a single form of the enzyme. The possible existence of multiple forms of epoxide hydratase is discussed.

Hepatic microsomal epoxide hydratase (EC 4.2.1.63) catalyses the bio-transformation of a wide variety of aromatic and olefinic epoxides [1]. The primary role of this enzyme appears to be the inactivation of potentially dangerous cytoxic or carcinogenic epoxide metabolites [2], although recent evidence has also suggested that this enzyme may also accelerate the rate of formation of very carcinogenic benzo (a) pyrene metabolites [3, 4].

Differential induction of epoxide hydratase activity towards styrene oxide by phenobarbital and 3-methylcholanthrene [5] suggests the possible existence of multiple forms of epoxide hydratase.

Multiple forms of other microsomal enzymes, such as cytochrome P-450 have been detected in rat liver [6]. The molecular weights (possibly subunit) vary between 47,000–60,000 and the quantities of these multiple forms appear to be selectively induced by 3-methylcholanthrene or phenobarbital [6]. Each of the cytochrome P-450 forms isolated may be responsible for postion–specific oxygenation of benzo (a) pyrene [7] and steroids [8]. Multiple forms of epoxide hydratase, if they exist at all, have not yet been observed.

We have isolated the enzyme(s) from drug-treated rat livers by the procedure developed in this laboratory [9] in an attempt to determine whether different forms of the enzyme can be selectively induced by 3-methylcholanthrene or phenobarbital. Further we have attempted to determine any structural differences by immunochemical analysis. The results which are reported suggest that only molecular weight species of epoxide hydratase could be isolated from xenobiotic-pretreated rat livers. The increase of enzyme activity by phenobarbital appears to be due to a selective increase in epoxide hydratase protein content of endoplasmic reticulum.

During the preparation of this manuscript Levin's group have described some immunological studies similar to those reported in this paper (W. Levin, personal communication). Some of this data is referred to here by kind permission of Dr. Levin.

MATERIALS AND METHODS

Rats. Male Wistar rats (200–250 g) were used in all experiments. Food and water were available *ad lib.*

Drug-pretreatment of rats. Phenobarbital (2 g/l.) was added to drinking water 10 days before death. During this period the rats consumed approximately 100 mg/kg body weight each day.

3-Methylcholanthrene 0.5 ml of 50 mg/ml (v/v) suspension in arachis oil (Sigma London Ltd.) was injected intraperitoneally into Wistar rats (equivalent to 100 mg/kg body weight) daily for three consecutive days. Anaimals were killed 24 h after the final injection.

Assay of epoxide hydratase. Epoxide hydratase activity was determined by the method of Oesch [10], using [7-^{3}H]-styrene oxide (The Radiochemical Centre, Amersham) as substrate. One unit of enzyme activity is defined as 1 nmole of styrene glycol formed/min.

Protein concentrations were determined by the method of Dulley and Grieve [11] using bovine serum albumin as standard.

Purification of epoxide hydratase. Liver microsomal pellets were obtained by the method previously described [12]. Epoxide hydratase activity was solubilised using the detergent Lubrol 12A9 (ICI Organics Division, Manchester, U.K.) and subsequently purified by two successive ion exchange chromatographic steps [9].

Sodium dodecyl sulphate gel electrophoresis. Gel electrophoresis was performed by using 7.5% (w/v) polyacrylamide gels in the presence of 0.1% sodium

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dodecyl sulphate at 20°, pH 7.2 and a constant 60 V (6 mA per gel). The proteins were stained with Coomassie Blue (Sigma London Ltd.) as previously described [13].

Immunochemical analysis. Antiserum was raised in White Lop-Eared Rabbits by subcutaneous injection of epoxide hydratase purified from untreated rat livers. Equal volumes of pure protein (0.5 mg/ml) and Freund's complete adjuvant (Grand Island Biological Company, NY, USA) were mixed into a fine emulsion. This mixture (1 ml) was injected into a rabbit. After four weeks a second identical quantity of protein mixture was injected into the rabbit. Two weeks later 20 ml of blood was collected from the ear vein and allowed to clot overnight at 4°. Clear antiserum was obtained after removal of the clot and blood cells by centrifugation at 1000*g* for 10 min.

Serum obtained prior to injection of epoxide hydratase did not contain any immunoglobulins capable of reacting with epoxide hydratase. Antiserum obtained following injection of epoxide hydratase contained immunoglobulines which reacted specifically with only the epoxide hydratase protein present in a crude enzyme preparation [9].

Immuno-chemical analyses were performed by the Ouchterlony double diffusion technique in 1% agar [14].

RESULTS

Effect of drug-pretreatment on epoxide hydratase activity. Epoxide hydratase activity in microsomal pellets obtained from several untreated or drug-treated rat livers, was solubilised by gentle homogenisation in a buffered solution containing 1% (w/v) Lubrol 12A9 detergent [9]. In each case more than 90 per cent of the enzyme activity present in the detergent-treated microsomal suspension was retained in 105,000 *g* supernatant fractions after centrifugation for 60 min. Thus the enzyme is solubilised to a similar extent by this procedure from untreated or drug-treated livers.

The epoxide hydratase activities of these maximally activated Lubrol-soluble supernatants were assayed using styrene oxide as substrate, to enable assessment of the effect of both phenobarbital and 3-methylcholanthrene pretreatment of the animals without inherent latency problems (see ref. 15). The results in Table 1 show that the epoxide hydratase activity of the phenobarbital-treated liver preparations was increased some 2.6-fold, whereas the activity of similar preparations from 3-methylcholanthrene-treated rats was unchanged.

Purification of epoxide hydratase from drug-treated rat livers. Epoxide hydratase was subsequently purified from a number of untreated or drug-treated rat livers. Purification of representative samples from each group of animals is shown in Table 2.

Epoxide hydratase specific activity some 39- or 49-fold in control and 3-methylcholanthrene-treated animals to levels of 221 and 291 units/mg protein respectively, although final specific activity values are slightly lower than those previously reported by this laboratory [9]. Epoxide hydratase from phenobarbital-treated rat liver required less increase in specific activity, only 24-fold, to reach a comparable state of purity. In fact the amount of purified enzyme obtained from approx. 300 mg of protein in the lubrol-soluble supernatant fractions of these control, phenobarbital or 3-methylcholanthrene pretreated rats was 1.4, 4.2 and 0.7 mg of epoxide hydratase protein respectively. These results would be expected if phenobarbital pretreatment had selectively increased epoxide hydratase content of the endoplasmic reticulum relative to the majority of microsomal proteins. Indeed, glucose 6-phosphatase specific activity is often decreased following phenobarbital pretreatment [16].

SDS-gel electorphoresis. Epoxide hydratase has been purified to a comparable state of purity from each group of animals when based on specific activity measurements. SDS polyacrylamide gel electrophoretic analysis was used to check for comparable purity of the

Table 1. The effect of the administration of phenobarbital and 3-methylcholanthrene on liver epoxide hydratase activity

Treatment	Specific activity (nmoles styrene glycol formed/min/mg protein)	Change of Activity (%)
None(8)	5.4 ± 0.7	100
Phenobarbital (6)	15.0 ± 1.4	263
3-Methylcholanthrene (4)	5.9 ± 0.8	109

Figures in parentheses show the number of experimental animal livers used.
Results are expressed as the mean ± S.E.M.

Table 2. Purification of epoxide hydratase from xenobiotic-pretreated rat livers

Purification step	Control		Phenobarbital		3-Methylcholanthrene	
	Specific activity	Yield	Specific activity	Yield (%)	Specific activity	Yield (%)
Lubrol-soluble supernatant	5.7	100	16.5	100	5.9	100
DEAE–cellulose eluate	55.1	56	178.5	40	61	30
CM–cellulose eluate	221.3	26	392.3	24	291	10

Epoxide hydratase specific activity is expressed as nmoles styrene glycol formed/min/mg protein.

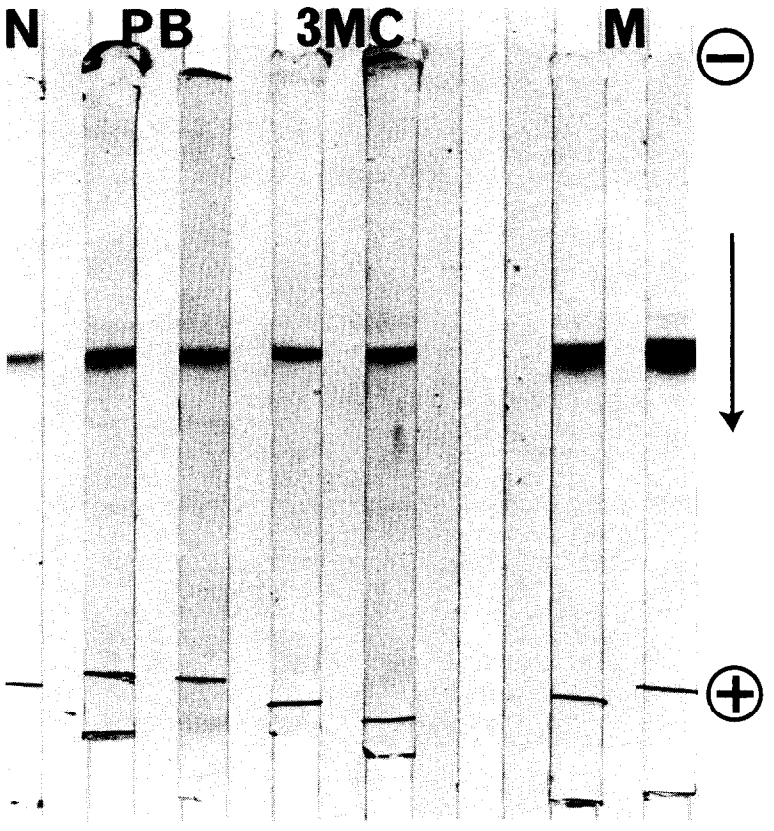

Fig. 1. Sodium dodecyl sulphate polyacrylamide gel electrophoretogram of purified epoxide hydratase from xenobiotic-pretreated rat livers. Disc electrophoresis was performed with 7.5% polyacrylamide gels (7 cm ×0.6 cm) in the presence of 0.1% sodium dodecyl sulphate (see Materials and Methods). Gel N, purified epoxide hydratase (4 μg of protein) from untreated rat. Gels PB, purified epoxide hydratase (10.7 μg of protein) from phenobarbital-pretreated rat liver. Gels 3-MC, purified epoxide hydratase (6 μg of protein) from 3-methylcholanthrene-pretreated rat liver. Gels M, mixture of equal amounts of purified epoxide hydratases from untreated rat liver and phenobarbital- and 3-methylcholanthrene-pretreated rat livers.

enzyme in each of the final preparations obtained. Gels stained for protein revealed that only a single major protein band was present in each case (see Fig. 1). Two very faint minor protein bands are contaminants in the purified fraction from 3-methylcholanthrene- and phenobarbital-treated rat livers. The mobility of the major protein bands of purified epoxide hydratase preparations from normal and xenobiotic pretreated livers are very similar. To confirm that the mobilities and hence molecular weights (possibly subunit) of the enzyme were the same, equal volumes of the three different preparations were mixed, denatured in the presence of SDS, before analysis by polyacrylamide gel electrophoresis. Gels 6 and 7 (Fig. 1) show the result of this experiment. Since no extra protein staining bands are visible all these preparations of epoxide hydratase appear to exhibit the same mobility and hence molecular weight on SDS-gel electrophoresis. The molecular weight in each case is approximately 50,000 [9].

Immunochemical analysis. In an attempt to confirm the identity of the proteins isolated from each experimental group of livers, we have investigated the immunochemical properties of the enzyme(s) by Ouchterlony double diffusion analysis. Antiserum raised in rabbits against epoxide hydratase purified from untreated rat livers (see Materials and Methods) was allowed to react with epoxide hydratase antigens obtianed from the other two xenobiotic-pretreated livers using the double diffusion analysis method. The results in Fig. 2 show that a single, sharp, immunoprecipitin line was visible. The continuity of this line indicates the identical immunological reaction which occurs between the antiserum and epoxide hydratase proteins purified from untreated or xenobiotic-pretreated rat livers.

DISCUSSION

Epoxide hydratase activity of rat liver is increased by pretreatment with phenobarbital as has been previously reported [5, 17, 18]. However 3-methylcholanthrene treatment of rats failed to increase the level of epoxide hydratase activity when measured with styrene oxide as substrate. Oesch [17] reported that 3-methylcholanthrene induced epoxide hydratase activity 2-fold in male Sprague–Dawley rat liver, although recently, further evidence has shown that an increase of epoxide hydratase activity cannot be detected using styrene

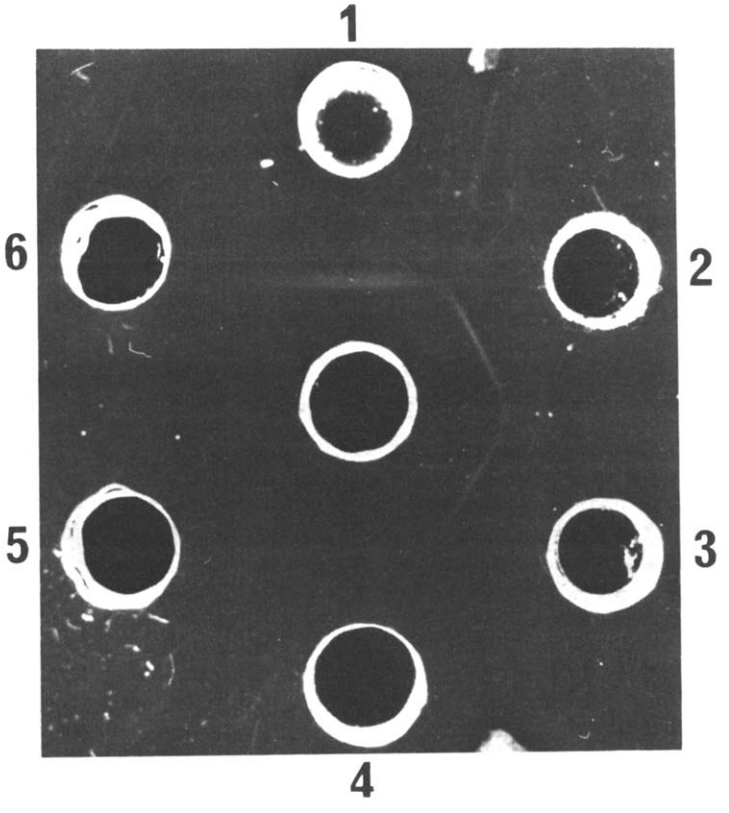

Fig. 2. Ouchterlony double-diffusion analysis of purified epoxide hydratase isolated from livers of xenobiotic pretreated rats. The experiment was performed using 1% agar plates, which were allowed to develop for 48 h at 15° before photography. Wells contained: centre, antiserum raised against pure epoxide hydratase from rat liver; 1, epoxide hydratase isolated from phenobarbital-pretreated rat liver; 2, epoxide hydratase isolated from control rat liver; 3, epoxide hydratase isolated from 3-methylcholanthrene-pretreated rat liver; 5, buffer blank; 4 and 6, empty.

oxide as substrate following treatment of rodents with 3-methylcholanthrene [6, 18–20], in agreement with our result.

Phenobarbital pretreatment of animals caused a stimulation of epoxide hydratase activity towards styrene oxide when measured in detergent-solubilised preparation from hepatic microsomes. This solubilisation procedure effectively released any latent epoxide hydratase activity present in the hepatic microsomal fraction. Thus it would appear unlikely that the stimulation of enzyme activity is due to any alteration of the latency of the enzyme.

A smaller increase of relative purification value was required to enable complete isolation of the enzyme from phenobarbital-treated rat liver. Examination of the purified epoxide hydratase from untreated or xenobiotic-pretreated animals suggests that a similar enzyme had been isolated from all sources exhibiting a similar specific activity. Thus the increased epoxide hydratase content of endoplasmic reticulum from phenobarbital-treated rat livers, would appear to be due to accumulation of only one molecular form of enzyme protein, although SDS gel electrophoresis of the purified enzyme is not capable of discriminating proteins of very similar molecular weight. This result is not too surprising, as a previous search for multiple forms of the enzyme preformed by measurement of the activity of the purified enzyme towards different substrates in the presence or absence of chemical or immunological inactivators, concluded that at least eight substrates were hydrated by the same enzyme purified to apparent homogeneity [21–23]. A similar immunological study to that presented here was recently produced by Dr. Levin's group. Antibody prepared in sheep against epoxide hydratase from phenobarbital-treated rats was reacted with liver microsomal enzyme obtained from control rats or rats pretreated with phenobarbital, 3-methyl-cholanthrene or pregnenolone -16x- carbonitrile. In all cases a single immunopreciptin line exhibiting identity with purified epoxide hydratase from phenobarbital-pretreated rats was observed (W. Levin, personal communication) [23].

Further, in this laboratory we have obtained evidence that similar molecular weight species of epoxide hydratase can also be purified from mouse liver [24], guinea pig liver, human liver and rat kidney (Hasani and Burchell, manuscript in preparation). Different forms of epoxide hydratase, however, were detected in different species using immunological techniques (Hasani and Burchell, manuscript in preparation) and again Levin's group have recently obtained some similar results (W. Levin, personal communication) [23].

The intriguing question raised by the report of Bresnick *et al.* [18] of a small selective induction of epoxide hydratase activity towards only certain substrates following exposure to 3-methylcholanthrene, is not necessarily indicative of the presence of different multiple forms of the enzyme. Certainly phenobarbital stimulated epoxide hydratase activity equally, towards all substrates tested [18].

Possibly 3-methylcholanthrene may alter the membrane microenvironment of epoxide hydratase by facilitating its interaction with certain substrates. Davison and Willis [25] have shown that 3-methylcholanthrene treatment of rats changes the fatty acid composition of rat liver microsomal phospholipids, which will affect their physical properties. Although epoxide hydratase is apparently not completely dependent upon phospholipids for activity, nevertheless alteration of the content and type of endoplasmic reticulum phospholipids could affect substrate interaction with the enzyme protein [26]. The activity of epoxide hydratase is demonstrably susceptible to alteration of the membrane micro-environment as shown by the ability of simple detergent treatment to increase activity with styrene oxide as substrate [15]. Thus it would appear worthwhile to study further membrane-derived latency of epoxide hydratase to determine whether this latency is responsible for different activities towards different substrates.

There appears to be only one form of epoxide hydratase which can be isolated from rat livers. Phenobarbital regulates epoxide hydratase activity by selectively increasing the enzyme protein content in hepatic endoplasmic reticulum, although other control mechanisms may operate through effects of the membrane environment on pre-existing enzyme protein. Although these data are consistent with the concept of a single form of rat liver epoxide hydratase they do not exclude the presence of multiple forms having similar molecular weights and similar antigenicities.

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