Biochemical Pharmacology, Vol. 34, No. 11, pp. 1987–1991, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

COMPARISON OF KIDNEY, LUNG AND LIVER COUMARIN 7-HYDROXYLASES IN PHENOBARBITAL PRETREATED DBA/2J AND C57BL/6J MICE

P. KAIPAINEN* and M. LANG†

Department of Physiology and †Department of Pharmacology and Toxicology, University of Kuopio, P.O.B. 6, 70210 Kuopio 21, Finland

(*Received* 21 *May* 1984; *accepted* 10 *December* 1984)

Abstract—In this paper we describe catalytic and immunological properties of coumarin 7-hydroxylase, a cytochrome P-450 dependent enzyme activity, in the liver, kidney and lung of C57BL/67 and DBA/2J mice. Coumarin 7-hydroxylase activity was higher in D2 than in B6 mice in all three organs. For both strains of mice, liver had the highest enzyme activity when expressed per milligram of microsomal protein. However, when expressed per nmole cytochrome P-450 there was no difference in the enzyme activity between the tissues. Inhibition of microsomal coumarin 7-hydroxylase by antibodies previously developed in our laboratory against a cytochrome P-450 fraction from D2 and B6 mouse liver, associated with coumarin 7-hydroxylase, occurred as follows. In D2 mice both antibodies caused approximately 50% inhibition of the enzyme activity of all three organs. In B6 mice, however, the only organ where considerable inhibition took place was the liver, and only when antibody against B6 cytochrome P-450 was used. Ouchterlony immunodiffusion analysis revealed a 100% crossreactivity between the two strains of mice when similar organs were compared. The 100% crossreactivity was also found between the liver and lung in both strains of mice. However, only a 50% crossreactivity was found between kidney and liver or kidney and lung in B6 and between the kidney and lung in D2. The data demonstrate interorgan and interstrain differences in the immunological and catalytical properties of cytochrome(s) P-450 catalysing coumarin 7-hydroxylation.

A genetic difference is known to exist among certain inbred strains of mice, including D2 and B6, in their capacity to metabolize coumarin to 7-hydroxycoumarin [1, 2]. In our previous work we have shown that the D2 mice (having a high capacity for coumarin 7-hydroxylation) have a liver microsomal cytochrome P-450 fraction with a high specific activity towards coumarin 7-hydroxylation. An immunologically identical cytochrome P-450 fraction was also found in the livers of B6 mice (having a low capacity of coumarin 7-hydroxylation) but it was shown to have a V_{max} only 50% and a K_m value 10-fold higher than that of D2; although it was also quite specific towards coumarin 7-hydroxylation [3, 4].

To date, all experiments on coumarin 7-hydroxylation have been carried out with liver preparations, and it is not known whether the genetic difference in the enzyme activity also exists in extrahepatic tissue. To answer this question we, in the present work, have compared the catalytic and immunological properties of coumarin 7-hydroxylase in the liver, kidney and lung of D2 and B6 mice. The mice were pretreated with phenobarbital because it is known to increase the enzyme activity and thus stress the genetic difference, at least in the liver.

MATERIALS AND METHODS

Chemicals. Chemicals were purchased from the following sources: Coumarin and 7-hydroxycoumarin from Aldrich Chemical Co., Milwaukee, WI, U.S.A., Sodium phenobarbital from Merck, Darmstadt, Germany, NADPH was from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were reagent grade.

Animals. The animals used were 4-week-old C57BL/6J and DBA/2J male mice and obtained from Laboratory Animal Center of University of Kuopio, Finland. All experiments were carried out using phenobarbital-pretreated animals. Sodium phenobarbital was given to mice i.p. as a 0.9% sodium chloride solution, pH 8.3. On the first day the dose was 30 mg/kg and on the following four days, 60 mg/kg.

Preparation of microsomes. Mice were killed (fifth day) by decapitation and their livers, lungs and kidneys were removed immediately. The organs were homogenized in 4 volumes of ice cold 150 mM KCl containing 10 mM EDTA, pH 7.4. The 9000 g supernatant of the homogenate was centrifuged at 105,000 *g* in order to harvest the microsomal fraction. After washing, microsomes were homogenized in 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM EDTA, and stored in −60° for further use.

Assay of coumarin 7-hydroxylase activity. The determination of coumarin 7-hydroxylase activity was carried out according to Aitio [5], using coumarin in place of ethoxycoumarin as a substrate. We used NADPH instead of regenerating system to start reaction. Our incubation mixture contained 1 mM coumarin, 100 mM potassium chloride, pH 7.4, 2 mM magnesium chloride, 0.05–0.5 mg microsomal protein, 3 mM NADPH and the total volume of the

* To whom correspondence should be addressed.

Table 1. Cytochrome P-450 contents and coumarin 7-hydroxylase activities of DBA/2J and C57BL/6J mouse liver, kidney and lung microsomes (the figures represent the average ± S.E.M. value of 20 individuals)

		Cytochrome P-450 (nmoles/mg protein)	Enzyme activity (pmoles/min) (/mg protein)	(/nmole P-450)
Liver	D2	1.4 ± 0.2	600 ± 33	428 ± 23
	B6	1.2 ± 0.2	150 ± 15	125 ± 13
Kidney	D2	0.10 ± 0.02	51 ± 5	510 ± 50
	B6	0.10 ± 0.02	14 ± 2	140 ± 20
Lung	D2	0.06 ± 0.01	35 ± 4	583 ± 67
	B6	0.06 ± 0.01	25 ± 3	417 ± 50

All animals were treated with phenobarbital.

mixture was 0.5 ml adjusted with distilled water. The mixture was incubated at 37° for 20 min and then 0.5 ml 0.31 M TCA was added to stop the reaction. Finally, 4 ml 1.6 M NaOH-glycine buffer, pH 10.3, was added and fluorescence was measured with a Perkin–Elmer MPF-43A fluorescence spectrophotometer. The emission wavelength was 440 nm and excitation, 390 nm. Standardization was made by using 7-hydroxycoumarin.

Other assays. Protein was measured as described by Lowry *et al.* [6]. The microsomal cytochrome P-450 content was determined according to Omura and Sato [7].

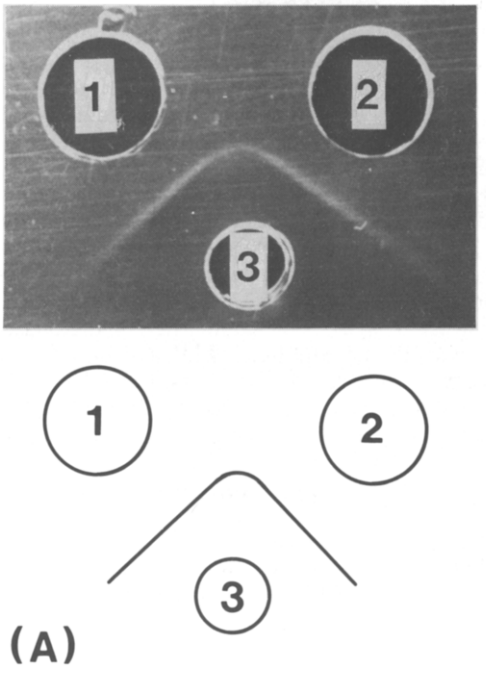
Immunological techniques. The cytochrome P-450 fraction with high specific activity towards coumarin 7-hydroxylation was purified from male, phenobarbital-pretreated, D2 and B6 mice, as described in detail by Kaipainen *et al.* [4]. Antibodies were raised in adult female New Zealand white rabbits according to the protocol of Negishi and Nebert [8]. Immunoglobulin G fractions from preimmune and immune female rabbits sera were prepared by ammonium sulphate fractionation and were finally dissolved in 100 mM potassium phosphate buffer, pH 7.5, for further use. When the mono-oxygenase complex was reconstituted by the antigens and used for enzyme-activity determinations, the antibodies inhibited the coumarin 7-hydroxylase by almost 100% [4]. In double diffusion analysis we used the general procedure of Thomas *et al.* [9]. The 1%-agarose plates contained 0.2% (w/v) Emulgen 911 prior to the addition to the samples wells. After solubilization microsomal fractions, from a pool of several individuals, were centrifuged at 105,000 *g* for 1 hr and the supernatant was used for the double-diffusion analyses. In D2 and B6 liver we applied 20–30 μg protein to the surrounding wells and about 100 μg antibody to the center well. In the kidney and lung, because of the low amounts of microsomal cytochrome P-450, we used the highest possible amount of protein in order to get obvious precipitin lines.

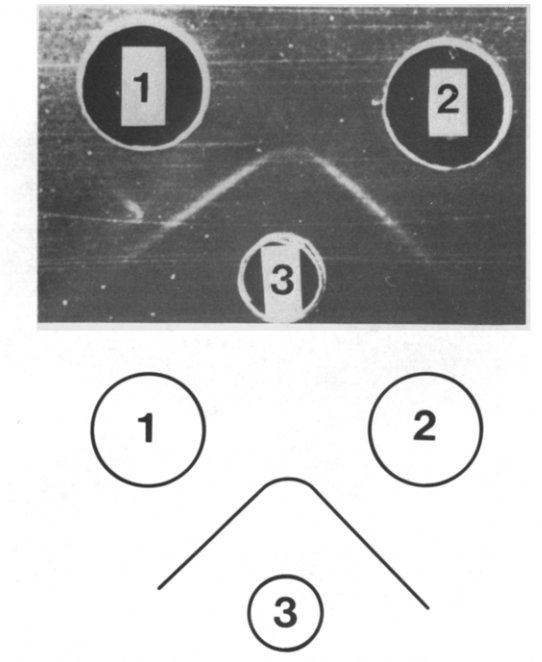
Inhibition studies. Inhibition of microsomal coumarin 7-hydroxylase activity by the antibodies was carried out by preincubation of the pool of microsomes from several individuals with the antiserum for 15 min at room temperature prior to starting the reaction. In control experiments similar incubation with preimmune serum was carried out. After preincubation enzyme determinations were carried out as described above.

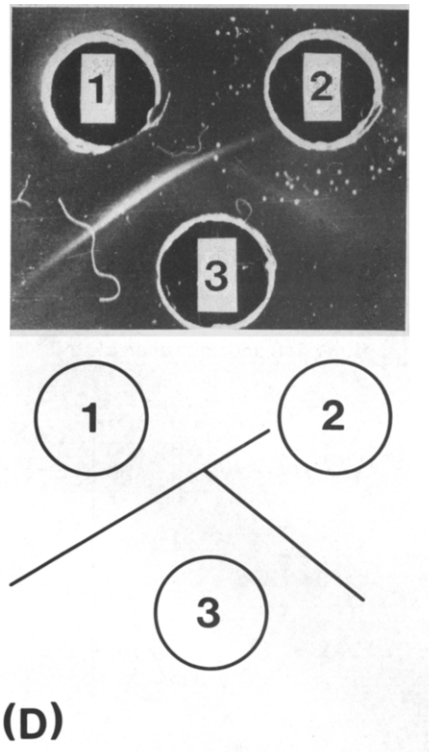
RESULTS

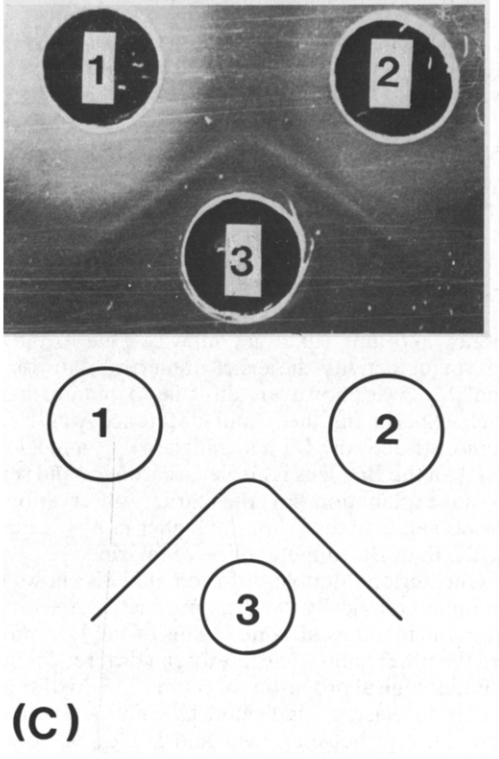
Microsomal cytochrome P-450 contents and coumarin 7-hydroxylase activities are shown in Table 1. The enzyme activity for D2 mice is higher than for B6 in all three organs. When the activity of coumarin 7-hydroxylase activity is expressed per microgram of microsomal protein, it is highest in the liver. However, when expressed per nanomole of cytochrome P-450, the activity for D2 is in the same range for all three organs. For B6 in this case, however, the highest activity is found in the lung.

Ouchterlony double-diffusion analyses, as shown in Fig. 1, demonstrates the immunoprecipitation of the coumarin 7-hydroxylase-specific cytochrome P-450 from livers, lungs and kidneys of D2 and B6 mice. A 100% crossreactivity between the two strains of mice can be observed for the same organ. A 100% crossreactivity (although not shown in the picture) was also found between lung and liver cytochrome P-450s. In B6 mice only a 50% crossreactivity can be seen between liver and kidney (Fig. 1D) and between lung and kidney (Fig. 1E). In D2 mice the 50% crossreactivity was found only between lung and kidney (not shown in the figure). The result of the









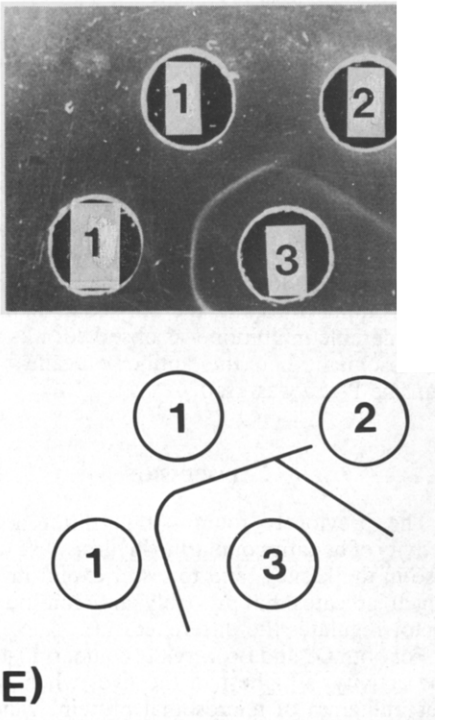


Fig. 1. Ouchterlony double-immunodiffusion analysis of coumarin 7-hydroxylase-specific cytochrome P-450 from livers, kidneys and lungs of D2 and B6 mice (a pool from several individuals). Interaction between the B6 (2) and D2 (1) mice are shown in the liver (A), lung (B) and kidney (C). Antiserum is applied in well no. 3. Same result was obtained with both antibodies. Immunoprecipitation between liver (1) and kidney (2) is shown in (D) and between lung (1) and kidney (2) in (E) for B6 mice. Antibody is applied in well no. 3. For more details see Materials and Methods.

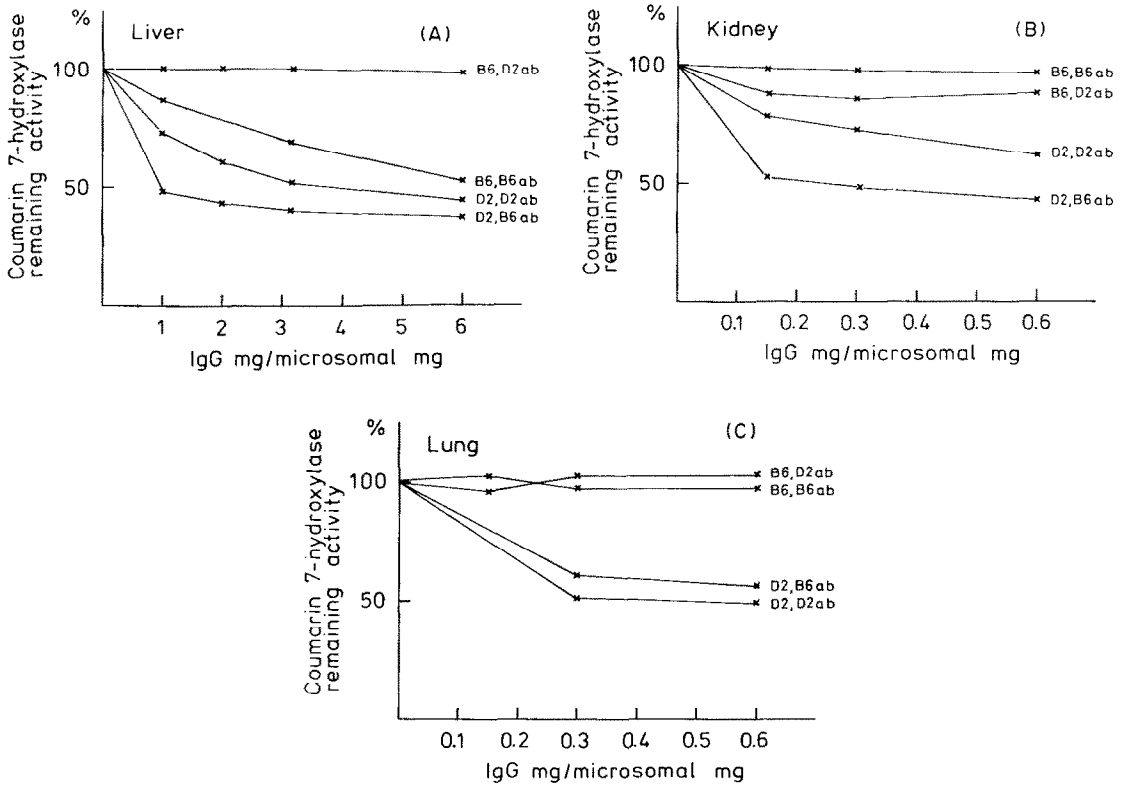


Fig. 2. Inhibition of coumarin 7-hydroxylase activity in D2- and B6-mouse liver, kidney and lung microsomes (a pool from several individuals) by antibodies against coumarin 7-hydroxylase specific cytochrome P-450 from D2 and B6 mice. Control activities were following: D2 liver = 580, B6 liver = 130, D2 lung = 35, B6 lung = 20, D2 kidney = 50 and B6 kidney = 14. The enzyme activity is expressed as pmoles 7-hydroxycoumarin formed/min/mg protein. For more details see Materials and Methods.

Ouchterlony double-diffusion analyses was identical with both antibodies.

Inhibition of microsomal coumarin 7-hydroxylase activity by the antibodies is demonstrated in Fig. 2. In D2 mice we can see that the enzyme activity is inhibited by 40–60% in all three organs by both antibodies. In B6 mice, however, the inhibition pattern is quite different: the only combination where considerable inhibition was observed, was with liver microsomes when the antibody against B6 cytochrome P-450 was used.

DISCUSSION

The previously found strain difference in the activity of hepatic coumarin 7-hydroxylase was found also in the kidney, and to lesser extent in the lung, which indicates that probably an organ-independent factor regulates the difference.

For both D2 and B6 mice the coumarin 7-hydroxylase activity is highest in the liver when calculated per milligram of microsomal protein, which is not surprising because this is the case for most of the known reactions catalysed by cytochrome P-450(s).

On the other hand, it is noteworthy that when the enzyme activity is expressed per nanomole of cytochrome P-450, it is at least as high or even higher in the kidney or lung than in the liver. This indicates that the relative amount of those cytochrome P-450(s) catalysing the 7-hydroxylation of coumarin of the total cytochrome P-450 pool in microsomes is at least as high in the kidney and lung as in the liver.

The previously found differences in the kinetic parameters (the K_m and V_{max}) of hepatic coumarin 7-hydroxylase of microsomes and purified proteins for D2 and B6 mice [4] could also be detected in the kidney and lung (data not shown). Due to the low enzymatic activity the exact numerical data for K_m and V_{max} was, however, difficult to obtain, but in each experiment the strain difference was clearly demonstrated: the D2 had a higher V_{max} and a lower K_m than the B6. This is also in accordance and serves as an explanation for the earlier observation by Wood and Conney [1] of the higher *in vivo* capacity of D2 than B6 to metabolize coumarin.

Ouchterlony double-diffusion analysis shows that an immunologically identical product is precipitated from microsomes of same organs of the D2 and B6. On the other hand some interorgan differences in the immunological properties of coumarin 7-hydroxylase seems to exist, as indicated by only 50% crossreactivity, e.g. between liver and kidney or between lung and kidney. It should be mentioned that by preliminary experiments the amounts of antibody and liver microsomes used in the immunodiffusion analysis were optimized. On this basis the amount of lung and kidney microsomes was then chosen so as the total amount of microsomal cytochrome P-450 in each well was as similar as possible.

Inhibition of microsomal coumarin 7-hydroxylase activity by the antibodies is interesting because it does not correlate with the immunodiffusion data: both antibodies form immunoprecipitin lines in case of all organs from both D2 and B6 but the enzyme activity was inhibited in B6 only in liver, and only with the antibody against its "own" cytochrome P-450 fraction. The inhibition and immunoprecipitation data thus indicate differences in the structures of the antigens because in case of D2 the antigenetic sites seem to be critical for the enzyme activity leading to inhibition, whereas in case of B6 they are not; the only exception being the combination of B6 liver and antibody against B6 cytochrome P-450.

In the inhibition studies the ratio of antibody to microsomal protein used varies depending on the organ. The reason for this is that a higher cytochrome P-450 content can be found in liver microsomes than in microsomes from kidney or lung.

From the above data we conclude: (1) that the genetic difference between D2 and B6 mice in coumarin 7-hydroxylase activity occurs not only in the liver but also in kidney and lung; and (2) that cytochrome P-450(s) catalysing the coumarin 7-hydroxylation in the liver, lung and kidney have interorgan and interstrain differences both in their immunological and catalytic properties.

Acknowledgements—This study was supported by the Finnish Academy of Natural Science, Grant No. 73120 122/81.

REFERENCES

1. A. W. Wood and A. H. Conney, *Science, N.Y.* **185**, 612 (1974).
2. A. W. Wood, *J. biol. Chem.* **254**, 5641 (1979).
3. M. Lang, M. Negishi, I. Stupans and D. W. Nebert, in *Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications* (Eds. E. Hietanen, M. Laitinen and O. Hänninen), p. 391. Elsevier Biochemical Press, Amsterdam (1982).
4. P. Kaipainen, D. W. Nebert and M. Lang, *Eur. J. Biochem.* In press (1985).
5. A. Aitio, *Analyt. Biochem.* **85**, 488 (1978).
6. D. K. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
7. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
8. M. Negishi and D. W. Nebert, *J. biol. Chem.* **254**, 11015 (1979).
9. P. E. Thomas, A. Y. H. Lu, D. West, J. Kawalek and W. Levin, *J. biol. Chem.* **251**, 1385 (1976).