fraction II (compound 2) was purified by column chromatography in the following order: QAE-Sephadex A-25 (formate, 2×21 cm), phosphocellulose (H^+, 1.7×21 cm) and Sephadex G-25 (3.5×43 cm). Yield: 2.1 mg. The compound was also oxidized to 6-carboxypterin by alkaline permanganate and identified as 6-hydroxymethylpterin: TLC (table); UV: λ_{max}, 273, 345 nm (H_2O); fluorescence: excited, 283, 368 nm, emission, 444 nm (H_2O); MS: M^+, m/e, 193 (2%). GC-MS (TMS): M^+, m/e, 409 (tris-TMS-6-hydroxymethylpterin, 100%). The pterin in the fraction III (compound 3) was purified by the following column systems and TLC: QAE-Sephadex A-25 (formate, 2.4×22 cm); CM-cellulose (H^+, 2.1×24 cm); TLC (Avicel SF, 0.1 M Na-phosphate buffer, pH 6.0) and Sephadex G-25 (3.5×43 cm). Yield: 1.1 mg. The compound was identified as isoxanthopterin: TLC (table); UV: λ_{max}, 285, 339 nm (H_2O); fluorescence: excited, 293, 350 nm, emission, 407 nm (H_2O); MS: M^+, m/e, 179 (100%); GC-MS (TMS): M^+, m/e, 395 (tris-TMS-isoxanthopterin, 71%).

It is well known that folate derivatives are widely distributed in higher plants and phosphate esters of 7,8-dihydro-D-erythro-neopterin and 6-hydroxymethylpterin are intermediates in the biosynthesis of dihydrofolate[5,6]. In spite of these facts, there have been few reports of the presence of unconjugated pterins in higher plants, though 6-hydroxymethylpterin has recently been isolated from the chloroplasts of spinach leaves[7]. By contrast, unconjugated pterins are widely found in both animals and microorganisms[8]. In the present work, in addition to 6-hydroxymethylpterin we have isolated D-erythro-neopterin and isoxanthopterin from the pericarps of *S. hassjoo*. This seems to be the first report demonstrating the presence of the latter 2 pterins in plant materials. *S. hassjoo* seems to be peculiar with respect to accumulation of nitrogenous compounds such as L-DOPA in very large quantities[9], stizolamine[1] and the unconjugated pterins described here. However, the biosynthesis and mechanism of accumulation of these compounds in the plant still remain to be studied.

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Non-linear kinetics of microsomal styrene monooxygenase after phenobarbital pre-treatment[1]

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Summary. Pretreatment of rats with phenobarbital, but not with 3-methylcholanthrene, induces liver microsomal styrene monooxygenase. Under these conditions the kinetic profile is not linear and can be divided into 2 distinct curves. Evidence is presented indicating that the combined treatment with phenobarbital and $CoCl_2$ destroy the high affinity enzyme, suggesting that the native cytochrome is less sensitive to the action of $CoCl_2$.

The first step in the metabolism of styrene is P-450 dependent epoxidation of the olefinic double bond by microsomal monooxygenases[3,4]. The resulting metabolite, styrene 7-8 oxide, is rapidly hydrated by microsomal epoxide hydrase to phenylethyleneglycol and then to mandelic acid, phenylglyoxylic acid, benzoic acid and hippuric acid. Since styrene 7,8 oxide is thought to be a toxic intermediate in styrene metabolism, the epoxide-forming enzyme system assumes an important role as the toxifying component. Several reports have been published by our group on the relationship existing in different animal species and in different organs between toxifying and detoxifying enzymes[5], describing kinetic parameters and the sensitivity of both enzymes to a variety of inducers and inhibitors[6]. In the course of our experiments we noticed that in rats, after in vivo pretreatment with phenobarbital, but not with 3-methylcholanthrene, the epoxide forming enzyme system was significantly induced and it showed non-linear kinetics.

This paper reports a detailed study of this phenomenon, resolving the monooxygenase activity into 2 components, 1 with high affinity for the substance and the other of lower apparent affinity. Evidence is also provided that the de novo synthesized cytochrome is much more sensitive to the action of $CoCl_2$ than the native cytochrome.

Materials and methods. Male CDF-COBS rats with a b. wt of 175±20 g were obtained from Charles River Italy (Calco, Como). The rats were given a commercial laboratory chow and water ad libitum and kept in air conditioned quarters (60% relative humidity, 22 °C) with a 12-h light-dark cycle. Animals were pretreated with phenobarbital i.p. at a dose of 80 mg/kg daily for 3 days; $CoCl_2$ was administered as a single dose of 40 mg/kg. In the combined treatments $CoCl_2$ was given 1 day after the last phenobarbital injection. After the last treatment rats were fasted for 16 h before sacrifice. Microsomes were isolated according to Kato and Takayanaghi[7] and their P-450 or P-448 content was measured according to Omma and Sato[8]. Styrene monooxygenase and epoxide hydrase activities were measured using styrene and styrene oxide, respectively, as substrates, as previously described[9]. Proteins were measured according to Lowry et al.[10]. Apparent Km and Vmax values were calculated by Woolf plots, the curves being resolved into 2 components as described by Greenlee and Poland[11].

Results and discussion. The Figure shows the Michaelis-Menten curves for styrene monooxygenase from control (curve No. 1) and phenobarbital pretreated (curve No. 2) rats. The kinetic profile in the phenobarbital pretreated animals is not linear and can be divided into 2 distinct

Apparent Vmax and Km values for microsomal styrene monooxygenase after different pretreatment

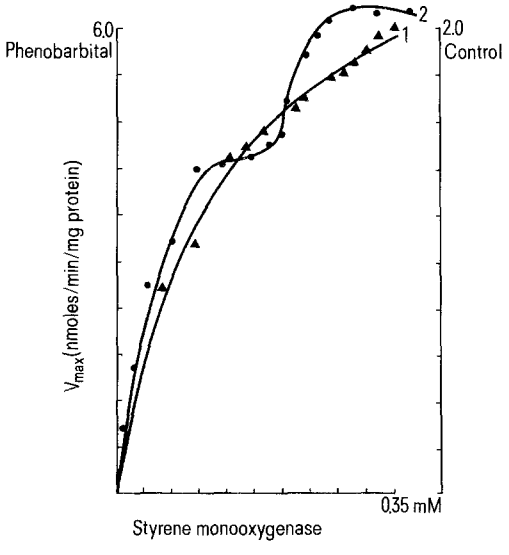
Treatment[a]	Cytochrome P-450 (nmoles/mg protein)	$Vmax_1$ (nmoles/min/mg protein)	$Vmax_2$ (nmoles/min/mg protein)	Km_1 (μM)	Km_2 (μM)
Control	0.68±0.05	2.30±0.08	-	0.09±0.01	-
Control + $CoCl_2$ (40 mg/kg)	0.44±0.07[b]	1.55±0.07[b]	-	0.19±0.09[b]	-
Phenobarbital (80 mg/kg)	2.10±0.11[b]	3.36±0.17[c]	5.78±1.19	0.06±0.01	0.21±0.07
Phenobarbital (80 mg/kg) + $CoCl_2$ (40 mg/kg)	0.91±0.09[b,d]	3.08±0.12[c]	-	0.08±0.01	-

[a] See materials and methods for pretreatment schedule; [b] $p<0.01$ compared with controls; [c] $p<0.05$ compared with controls; [d] $p<0.01$ compared with phenobarbital pretreated rats.

curves. The results are detailed in the table. Control animals show an apparent Km value of 0.09 μM and a Vmax of 2.30 nmoles/min/mg protein. In the induced animals the apparent Km values are 0.06 and 0.21 μM for high and low affinity curves respectively, and the Vmax values are 3.36 and 5.78 nmoles/min/mg protein. Phenobarbital pretreatment thus did not influence the apparent kM values, but only increases the Vmax value.

In our experimental conditions a single dose of $CoCl_2$, 40 mg/kg, destroyed about 35% of cytochrome P-450 giving 0.442 nmoles/mg protein compared with the control value of 0.680 nmoles/mg protein. Styrene monooxygenase activity after $CoCl_2$ treatment decreased proportionally, from 2.30 to 1.55 nmoles/min/mg protein. The apparent Km value was considerably higher, reaching 0.19 μM. The combination of both phenobarbital + $CoCl_2$ pretreatments causes the low affinity curve to disappear, but no substantial changes were observed in the animal treated with phenobarbital alone. In fact the apparent Km values for phenobarbital-only and for phenobarbital + $CoCl_2$ pretreated animals (0.06 versus 0.08 μM) and the Vmax values (3.36 versus 3.08 nmoles/min/mg protein) are not significantly different.

The presence of a similar pattern of induction for the monooxygenase has already been reported by other authors. For instance Ullrich et al.[12], Lehrmann et al.[13] and more recently Greenlee and Poland[11] have shown that ethoxycoumarin O-dethylase activity in mouse hepatic microsomes was not linear and was resolvable into 2 components.



Michaelis-Menten plot of liver microsomal styrene monooxygenase from control (curve 1) and phenobarbital (curve 2) pretreated rats. Each point is the mean value of 4 different experiments.

They also affirmed that phenobarbital increased the activity of low and high affinity components whereas 3-methylcholanthrene did not affect the activity of either. Greenlee and Poland[11] reported that the inducibility to 3-methylcholanthrene was related to the strain of mice used and in fact, in their experiments, phenobarbital induced the high affinity species in the Vmax values of C57B1/6J and DBA/2J mice. 3-Methylcholanthrene induced the high affinity component in C57B1/6J mice but not in DBA/2J mice which are not responsive to polycylic hydrocarbons.

The presence of multiple forms of cytochrome P-450 that can be isolated from liver microsomes of various animals induced with phenobarbital and 3-methylcholanthrene has been reported by several authors[14–16]. Thus the synthesis of distinct enzymatic populations in microsomes after in vivo pretreatment with inducers may explain the presence of high and low affinity components.

The animal species, the rat, used in our experiments, behaves differently from the mouse, because the low affinity curve was not visible in control conditions. Moreover only the treatment with phenobarbital, but not with 3-methylcholanthrene (data not presented here), induced the low affinity component.

The combined treatment with phenobarbital and $CoCl_2$ increased the destruction of de novo synthesized cytochrome P-450 indicating that the native cytochrome is less sensitive to the action of $CoCl_2$.

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