Moreover, 4-HC pretreatment did not change significantly platinum levels, therefore ruling out increased intracellular platinum as the principal mechanism responsible for the synergism between 4-HC and cisplatin.

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In vivo and *in vitro* effect of phenytoin on rat hepatic mixed function oxidases

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Phenytoin, a potent antiepileptic drug, is still widely prescribed during pregnancy for seizure control. A variety of congenital abnormalities, both in human [1, 2] and experimental animals [3, 4] have been observed following phenytoin treatment. Apart from prenatal exposure, young children are also exposed to phenytoin due to the use of this drug for the management of epilepsies and psychiatric ailments. Besides severe neurotoxicity, hepatotoxicity has also been observed in phenytoin treated individuals, though the fatal hepatic reactions are rare. Hepatocellular degeneration and/or necrosis associated with granulomas and cholestatic injury in treated human subjects has been reported [5, 6]. Developing children under the age of two are at higher risk of hepatic dysfunction who are being

Table 1. Effect of phenytoin on the activity of rat liver aniline hydroxylase and aminopyrine *N*-demethylase at 4 and 12 weeks of age

	Aniline hydroxylase (pmoles *p*-aminophenol formed/min/mg protein)		Aminopyrine *N*-demethylase (nmoles formaldehyde formed/min/mg protein)	
	Control	Treated	Control	Treated
4 weeks		36.3 ± 2.6**		0.54 ± 0.08*
Male	134.1 ± 10.2	(73 ± 2%)	0.88 ± 0.07	(39 ± 2%)
		70.7 ± 4.2**		
Female	120.9 ± 8.5	(42 ± 1%)	0.58 ± 0.06	0.53 ± 0.03
12 weeks		68.3 ± 5.2*		
Male	97.6 ± 6.9	(30 ± 2%)	0.99 ± 0.06	0.89 ± 0.05
		51.7 ± 2.9**		
Female	83.9 ± 5.9	(38 ± 2%)	0.59 ± 0.03	0.53 ± 0.03

Each value is mean ± SE of six animals.
Significantly different compared to controls: *$P < 0.01$; **$P < 0.001$.
Percentage decrease denoted in parenthesis.

treated by multiple drug regime for their seizure disorder and other medical illnesses [7]. At present relatively little information is available on the effect of phenytoin on the neonates following direct exposure. Therefore, we have investigated the effect of phenytoin on the activity of aniline hydroxylase (AH*) and aminopyrine *N*-demethylase (APN-D) to see if early exposure to phenytoin could affect the capability of neonates to handle xenobiotics.

Materials and methods

Phenytoin was obtained from Parke-Davis (India) Limited. Aminopyrine HCl and aniline HCl were supplied from Merck and Co. (India), NADP, glucose-6-phosphate and $MgCl_2$ were obtained from Sisco Research Laboratories (Bombay, India) and all other chemicals used were of highest analytical grade.

One day old albino rat litters were obtained from Industrial Toxicology Research Centre, animal breeding colony and maintained on a commercial pellet diet (Hindustan Lever Ltd, Bombay) under standard laboratory conditions. The litter size was adjusted so that each mother had eight pups. Mothers were divided into two groups, each consisting of six litters. Pups of one group were administered phenytoin 40 mg/kg by oral gavage daily from day two of age for 14 days. The control pups were given equivalent amounts of distilled water in an identical manner. At the age of 4 or 12 weeks, six pups each from control and treated groups representing various litters were killed. Livers were dissected out, weighed and processed for the estimation of aniline hydroxylase and aminopyrine-*N*-demethylase.

Effect of phenytoin on AH and APN-D under *in vitro* conditions was studied by incubating the 9000 *g* supernatant of liver homogenates of 4-week-old rats directly with varying concentrations of phenytoin (10^{-7}–10^{-4} M).

Liver homogenates 20% (w/v) of control and phenytoin treated groups were prepared in 0.1 M phosphate buffer pH 7.4 containing 1.15% KCl, using an Arthur Thomas tissue grinder. The homogenates were subsequently centrifuged at 9000 *g* for 20 min to isolate the post mitochondrial supernatant. The activity of AH was assayed according to the method of Kato and Gillette [8] by measuring the intensity of blue colour at 620 nm in a spectrophotometer. Activity of APN-D was estimated by the method of Cochine and Axelrod [9] where the intensity of yellow colour of formaldehyde formed was measured at 415 nm as described by Nash [10]. Protein contents of the samples were estimated by the method of Lowry *et al.* [11] using bovine serum albumin as a reference standard. Linearity with time and enzyme concentration was demonstrated for various biochemical assays performed in this study.

The data were statistically analysed by the Student's *t*-test as described by Fischer [12] and $P < 0.05$ was considered to be significant.

Results and discussion

Phenytoin exposed rat pups did not show any sign of overt toxicity. The animals looked normal and showed no sign of dyspnoea, palpatation or increased rate of respiration. No significant difference was observed between the total liver weight of treated and control animals. Also there was no difference in the overall body weight gain between control and treated animals (data not shown).

The effect of phenytoin on mixed function oxidases in 4- and 12-week-old rats is shown in Table 1. Early neonatal exposure to phenytoin caused a decrease of 73% in males and 42% in females in the activity of AH which persisted up to adulthood (12 weeks) in both males (30%) and females (38%). The degree of inhibition was reduced in males at adult stage than that seen at 4 weeks of age. However in females, there was no such change in the degree of inhibition with respect to time. Activity of APN-D was inhibited only in males by 39% at 4 weeks of age which returned to normal values at 12 weeks of age. In females no change in APN-D activity was observed on exposure to phenytoin either at 4 or 12 weeks of age. The results indicate that early phenytoin exposure inhibits the activity of AH and APN-D in a sex dependent manner. The partial or complete recovery of the inhibited activity may be due to the maturation of the drug metabolizing enzymes with full expression of their activity with age.

Neonates are vulnerable to toxic manifestations of xenobiotics probably due to poorly developed metabolic and excretory mechanisms leading to inefficient detoxification and elimination. Administration of drug while the brain and hepatic tissues are still undergoing morphological and biochemical development causes alterations in developmental parameters which persist even after the drug is withdrawn and last into adulthood. The specific activities of APN-D and AH are almost negligible at birth but progressively increase in both sexes over a 5 week period. Phenytoin treatment at this critical stage of enzyme development may be responsible for the decreased enzyme activities.

Phenytoin under *in vitro* conditions inhibited the activi-

* Abbreviations: APN-D, aminopyrine *N*-demethylase; AH, aniline hydroxylase; HPPH-5, (hydroxyphenyl)-5-phenylhydantoin.

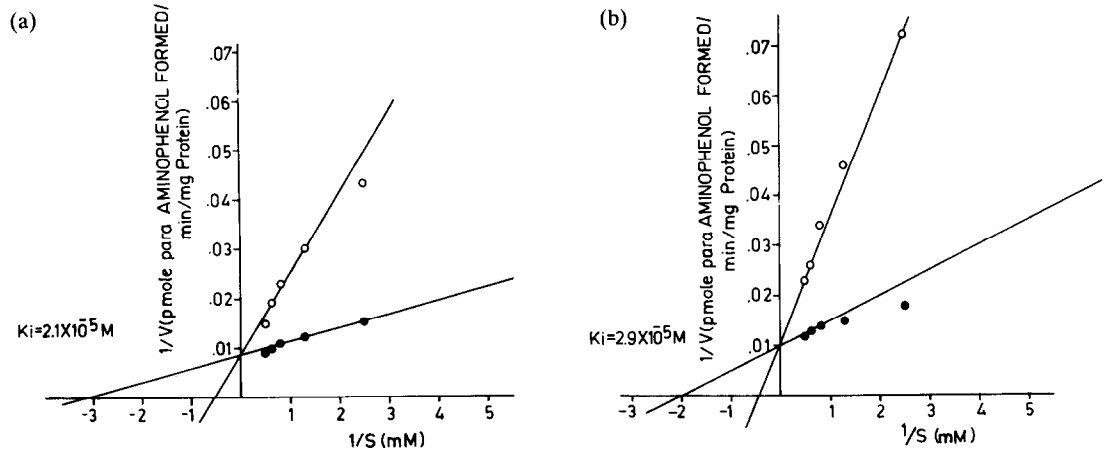

Fig. 1 (a) Lineweaver–Burk plot representing competitive inhibition of aniline hydroxylase activity by phenytoin in male rats. (○—○) With phenytoin, (●—●) without phenytoin. (b) Lineweaver–Burk plot representing competitive inhibition of aniline hydroxylase activity by phenytoin in female rats. (○—○) With phenytoin, (●—●) without phenytoin.

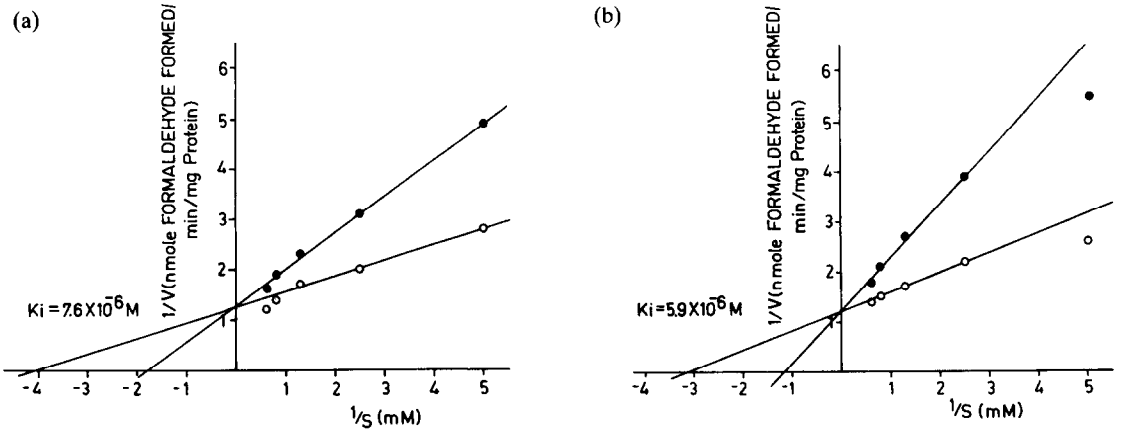

Fig. 2 (a) Lineweaver–Burk plot representing competitive inhibition of aminopyrine *N*-demethylase activity by phenytoin in male rats. (●—●) With phenytoin, (○—○) without phenytoin. (b) Lineweaver–Burk plot representing competitive inhibition of aminopyrine *N*-demethylase activity by phenytoin in female rats. (●—●) With phenytoin, (○—○) without phenytoin.

ties of AH and APN-D in a concentration dependent manner. The IC_{50} values were 79.2 and 31.62 μM for APN-D and AH respectively (Table 2). Lineweaver–Burk plots revealed that phenytoin inhibited AH competitively with an apparent K_i value of $2.1 \times 10^{-5} \pm 0.01$ M for males and $2.9 \times 10^{-5} \pm 0.02$ M for females (Fig. 1a and b). On the other hand Fig. 2a and b show that phenytoin inhibited APN-D competitively with a K_i value of $7.6 \times 10^{-6} \pm 0.04$ M for males and $5.9 \times 10^{-6} \pm 0.03$ M for females. Sex difference observed in K_i values were found to be statistically significant for both enzymes ($P < 0.01$ for AH and $P < 0.001$ for APN-D).

There is a difference in the pattern of maturation of the capacity for oxidation of type I and type II substrates in male and female animals [8, 13]. Males usually have higher APN-D activity in comparison to females and this could also be one of the possible reasons for the restoration of normal activity pattern of APN-D in males on maturation.

The observed inhibition of AH and APN-D activity both under *in vivo* and *in vitro* conditions indicate that phenytoin adversely affects the mixed function oxidase system. The normal low levels of the drug metabolizing enzymes present in neonates and their further inhibition by phenytoin may possibly result in raised levels of phenytoin leading to increased production of reactive metabolites involved in the toxic reactions [14, 15]. Both *in vivo* and *in vitro* studies have demonstrated that 5-(hydroxyphenyl)-5-phenylhydantoin (HPPH), a major metabolite of phenytoin markedly prolongs half-life of the drug by inhibiting its metabolic disposition [16–18].

HPPH is also known to inhibit the metabolism of both type I and type II substrates under *in vitro* conditions [19]. Interaction of HPPH with cytochrome P-450 may presumably be the reason for competitive inhibition of both enzymes observed under *in vitro* conditions. Our data indicates that neonatal phenytoin exposure causes inhi-

Table 2. Concentration of phenytoin required to inhibit drug metabolizing enzymes in liver by 50% (IC_{50})

Enzymes	IC_{50} Value*
Aniline hydroxylase	31.6 μM
Aminopyrine-*N*-demethylase	79.2 μM

* Average of three experiments in triplicate.

bition of both APN-D and AH which metabolize type I and II substrates respectively. Iinhibition of these enzymes may also impair the metabolism of various other drugs or xenobiotics which are inactivated by the same pathway rendering the developing organism more sensitive to drugs and chemicals.

In summary, present studies demonstrate that neonatal (2–14-day-old) phenytoin exposure (40 mg/kg/day orally) inhibited aniline hydroxylase and aminopyrine-*N*-demethylase in postnatally maturing animals, which persisted up to 12 weeks of age. *In vitro* studies showed that phenytoin inhibited the enzyme activities competitively. These effects were sex dependent. Inhibition of these enzymes may be responsible for reduced phenytoin metabolism and hepatic damage seen under clinical conditions.

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Muscarinic regulation of Ca^{2+} mobilization in a human salivary myoepithelial cell line

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Secretion of saliva is considered to be under the control of the autonomic nervous system [1, 2]. Studies concerned with the neuroreceptor regulation of salivary secretion have largely been confined to acinar cell preparations (e.g. Refs 1 and 3–5) or to excretory duct segments [6]. Myoepithelial cells represent an additional component of salivary and other exocrine glands [7]. While the exact function of these cells in secretory events is not clearly established, they are