phosphamide for the E3 line of rat embryo liver cells. There was an approximate 50 per cent reduction in the number of viable cells after exposure to the drug.

Using the more sensitive test of measuring cloning efficiency, shown in Table 2, there was a significant loss of viability when the rat embryo liver cell lines E3 and B1 were exposed to 2 μg/ml of cyclophosphamide. The BL line of rat embryo fibroblasts, however, was unaffected by the drug. Pretreatment of the E3 liver cells with the glucocorticoid dexamethasone at a concentration of 10^{-4} M for either 1 or 5 days before challenge with cyclophosphamide protected the cells from the toxic effects of the cyclophosphamide. Whether such treatment altered the activation of cyclophosphamide or protected the cells from the effects of the activation products is not yet established. Analogous experiments with phenobarbital were inconclusive, since incubation of E3 cells with this compound for more than several hours resulted in a variable loss of cloning efficiency in the absence of added cyclophosphamide.

In summary, two lines of rat embryo liver cells were shown to be sensitive to cyclophosphamide *in vitro*. Such culture systems may be useful in studying the pharmacology of this drug at a cellular level.

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Effects of some epoxides on aryl hydrocarbon hydroxylase activity

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Microsomal monoxygenase and epoxide hydrase are involved in the biotransformation of various toxic and carcinogenic compounds [1–7]. The former enzyme system is generally known to "activate" aromatic compounds into the more toxic or carcinogenic arene oxide (epoxide) derivatives, and the latter enzyme catalyzes the conversion of arene oxides to the less harmful dihydrodiols. The detoxification function of epoxide hydrase has recently been assessed by investigating the effects of some epoxide hydrase inhibitors, such as trichloropropene oxide (TCPO) and cyclohexene oxide, on chemically induced hepatic necrosis or carcinogenesis [8–10]. However, some of the results can not be explained by the inhibitory actions of these oxides on the epoxide hydrase activity. These compounds apparently also affect other enzyme systems. This communication deals with the effects of some epoxide compounds on the aryl hydrocarbon hydroxylase (AHH) activity, one of the catalytic functions manifested by the monoxygenase system.

The epoxides, 1,1,1-trichloropropene 2,3-oxide, 4-chlorophenyl 2,3-epoxypropyl ether and cyclohexene oxide were obtained from Aldrich Chemical Co. These compounds were added to the reaction mixture in 10–20 μl acetone and the solvent had no effect on the reaction. The AHH activity was assayed by measuring the phenolic fluorescent products according to previous procedures [11].

As shown in Fig. 1, TCPO inhibited the liver microsomal AHH activities of both mice and rats. While it inhibited the AHH activity of mouse microsomes at all concentrations, the compound apparently stimulated the AHH activity of rat microsomes at concentrations between 2 and 5 mM.

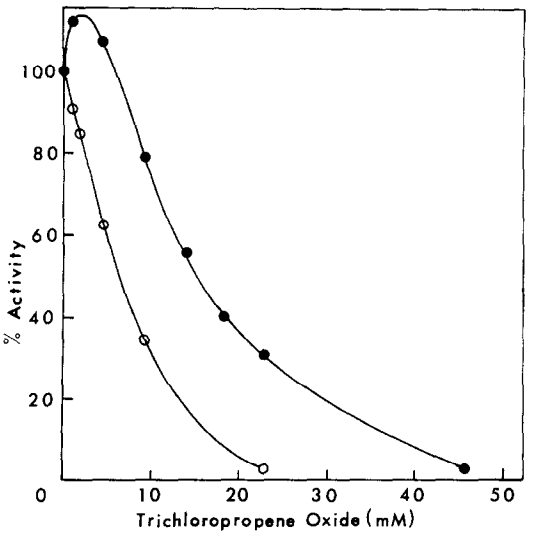

Fig. 1. Inhibition of aryl hydrocarbon hydroxylase by trichloropropene oxide. The assay mixture (1 ml) contained microsomes equivalent to 0·18 mg protein and the incubation time was 10 min. The activities in the absence of an inhibitor were 0·635 mole/min/mg for rat liver microsomes (●——●) and 0·628 nmole/min/mg for mouse liver microsomes (○——○).

Table 1. Effects of epoxide compounds on aryl hydrocarbon hydroxylase*

Epoxides	% Activity Mouse microsomes	Rat microsomes
Trichloropropene oxide (2 mM)	84	114
Chlorophenyl epoxypropyl ether (0·7 mM)	80	110
Cyclohexene oxide (5 mM)	82	135

* Assay conditions are similar to those of Fig. 1.

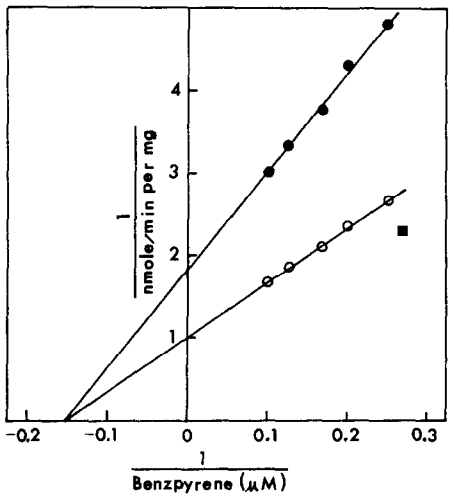

Fig. 2. Lineweaver–Burk plots of trichloropropene oxide inhibition. Assay conditions were similar to those of Fig. 1, except that 0·115 mg protein and an incubation time of 5 min were used. Key: O——O in the absence and ●——● in the presence of 5 mM trichloropropene oxide.

TCPO also inhibited the AHH activities of mouse skin homogenates as well as microsomes prepared from livers of beef and monkey. The pattern of inhibition was similar to that of mouse microsomes.

Two other epoxide hydrase inhibitors, chlorophenyl epoxypropyl ether and cyclohexene oxide, also have similar effects on the AHH system (Table 1). At a concentration of 0·7 mM, chlorophenyl epoxypropyl ether inhibited the mouse but elevated the rat enzymic activities. At higher concentrations, e.g. 2·5 mM, it inhibited both the mouse and rat AHH activities by approximately 50 per cent. Cyclohexene oxide is a weaker inhibitor of the hydroxylase system. At a concentration of 5 mM, it caused an 18 per cent inhibition of the mouse enzyme but a 35 per cent enhancement of the rat system. The increment in AHH activity may be a consequence of the blockade of epoxide hydrase, since an inhibition of the enzymic conversion to the dihydrodiol would enhance the nonenzymic isomerization of the arene oxide to phenolic products [12]. A direct stimulation of the initial oxygenation reaction by the epoxides is also possible. The molecular basis for the species differences remains to be investigated.

As shown in Fig. 2, TCPO inhibited the mouse microsomal AHH activity by a noncompetitive mechanism. The detailed mechanism of the inhibition is not known. Epoxides are reactive toward sulfhydryl groups. The inhibition, however, is not due to modification of the sulfhydryl groups of the NADPH-cytochrome *c* reductase. In the presence of 10 mM TCPO, inhibition of the reductase activity and denaturation of cytochrome P-450 were not observed. The inhibition of AHH by epoxides is probably not related to their inhibition of the epoxide hydrase, since these compounds are at least ten times more potent in inhibiting the latter than the former enzymes [4, 5, 8, 12].

Although the concentration of epoxides used in this investigation are higher than those used in some other inhibition studies, the levels may resemble the local concentration of the epoxides in some experiments *in vivo*. For example, the presently observed inhibitory action of TCPO is consistent with the finding that higher levels of TCPO inhibited the AHH-dependent binding of 3-methylcholanthrene to DNA [10] and decreased 3-methylcholanthrene-induced tumor formation [9]. The concept that epoxides are inhibitors of the monoxygenase system is also in agreement with the observations of Oesch *et al.* [8] that coadministration of cyclohexene oxide and chlorobenzene significantly reduced the rate of metabolism of the latter compound and prevented the hepatic necrosis caused by chlorobenzene. The inhibition of AHH by epoxides and the species differences of the inhibition have to be considered in future studies, especially with experiments *in vivo* in which the cellular local concentrations of the compounds may reach rather high levels.

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Reduction *in vivo* of (−)-nicotine-1′-*N*-oxide by germ-free and conventional rats

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To date, only a few studies on the reduction *in vivo* of *N*-oxides have been reported [1–4]. These investigations are at variance with regard to the site, extent and/or mechanism(s) of reduction of the limited range of compounds studied. Recently, Beckett *et al.* [5] reported reduction of (−)-nicotine-1′-*N*-oxide to (−)nicotine after oral administration of the oxide to human subjects. However, following intravenous administration of the compound no appreciable reduction could be observed. This was interpreted as being due to reduction by gut content, although no direct evidence was given to support this contention. In the present work we have re-examined the reduction of the oxide using germ-free as well as conventional rats in order to gain more insight into the problem.

Male Wistar Albino rats of nearly the same age and weighing approx. 200 g were housed in separate metabolic cages and had free access to a standard diet (Dixon's diet 86) and water. A single oral dose of (−)-nicotine-1′-*N*- oxide (1·5 mg base/kg body wt) or (−)-nicotine tartrate (1 mg base/kg body wt) was administered by gavage to each rat. Urine, free from faeces, was then collected in tubes surrounded by carbon dioxide ice for a period of 20 hr.

The germ-free rats of the Agus strain weighing approx. 150 g were transferred individually to metabolic cages immediately after arrival from the supplier. One millilitre sterile solution of (−)-nicotine-1′-*N*-oxide (0·4 mg base/ml) was given by gavage to each rat, and the urine was collected as described above for the conventional rats. The animals were then recontaminated with the microflora of the animal house by keeping them in cages containing faecal material from conventional rats for 3–4 weeks; the experiment was then repeated with a second dose of the *N*-oxide and the urine was similarly collected. The recontaminated animals, as well as the conventional rats, were then treated with antibiotics, and the administration of (−)-nicotine-1′-*N*-oxide was repeated 3 weeks after the last dose of the oxide. These animals thus served as their own control.

Antibiotic treatment consisted of 1 ml of the following mixture given orally twice daily for three consecutive days: 100 mg Tetracycline HCl, 100 mg Neomycin sulfate and 100,000 units Nystatin, suspended in 1% aqueous methyl cellulose solution. On the 4th day a similar dose of the antibiotic mixture was administered, followed after 4 hr by the administration of (−)-nicotine-1′-*N*-oxide. Four hours later an additional dose of the antibiotic mixture (1·5 ml) was given.

In an attempt to by-pass any possible intestinal reduction of the *N*-oxide, the drug was also administered intraperitoneally to the same conventional group of rats (0·8 mg base/kg body wt dissolved in 8·5 ml sterile normal saline) 3 weeks after the last oral dose of the drug. Urine samples were collected as described in the above-mentioned experiments. Each of the frozen urine samples from all the foregoing experiments was thawed and analysed for (−)-nicotine, nicotine-1′-*N*-oxide and cotinine by gas liquid chromatography as described by Beckett *et al.* [6].

It is clear from Table 1 that considerable reduction of (−)-nicotine-1′-*N*-oxide occurred following its oral or intraperitoneal administration (1·5-fold more by the oral route). The formation of (−)-nicotine following the i.p. administration is of particular interest as it strongly suggests the occurrence of hepatic and other tissue reduction of the *N*-oxide. In fact, Dajani *et al.* [7] have recently reported a profound hepatic and intestinal tissue reduction of (−)-nicotine-1′-*N*-oxide *in vitro* in the rat. It is worth noting that the foregoing experiments are in conflict with the studies reported earlier [5], probably because of species variation.

Contrary to expectation from the known effects of antibiotics on the gut flora, the amounts of (−)-nicotine formed by each rat as well as by the whole group after treatment with antibiotics was significantly more than the controls ($P < 0{\cdot}05$). It would seem that following the administration of antibiotics, changes in the number and composition of the gut flora must have occurred. These could have led to alterations in the intestinal motility and, in turn, to changes in the pH of the gut contents. Such changes would alter the rate and site of absorption of the administered drug as well as the rate and routes of biotransformation and excretion [8]. Alternatively, the increased excretion of (−)-nicotine could be due to an excessive over-growth of some reducing organisms not sensitive to the antibiotic mixture used. Despite the fact that the bacteriology of the gastro-intestinal tract content is known, a bacteriological study after the antibiotic treatment is needed to confirm this speculation.