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The effect of pretreating rats with 3-methylcholanthrene upon the enhancement of microsomal aniline hydroxylation by acetone and other agents

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Various compounds have been found to enhance the hydroxylation of aromatic amines by the hepatic microsomal fraction *in vitro*, following their addition to the reaction mixture [1, 2]. Anders [3] has suggested that the enhancing effect of acetone might be attributable to the presence of two aromatic amine hydroxylases in the microsomal fraction, one with a high substrate affinity and a low V_{max} which is inhibited by acetone, and the other with a low substrate affinity and a high V_{max} which is not inhibited by acetone and whose properties only become apparent when the former hydroxylase is inhibited. Vainio and Hänninen [4] found that acetone added to the microsomal fraction decreased the type II difference spectrum and increased the type I difference spectrum produced by aniline. They suggested a correlation between the increased type I spectral change and the enhancement of aniline hydroxylation by acetone. They also found that phospholipase C digestion of the microsomal fraction, which destroys the type I binding site [5], abolishes acetone enhancement of aniline hydroxylation [4].

The pretreatment of rats with polycyclic hydrocarbons such as 3-methylcholanthrene has been shown to diminish the type I difference spectrum produced by drugs and to decrease metabolism of type I substrates [6]. If acetone is producing its effects upon aniline hydroxylation by promoting the type I binding of aniline, then it might be expected that pretreating rats with 3-methylcholanthrene would result in a decrease in the ability of acetone to enhance aniline hydroxylation. Anders [7] has reported however that the aromatic hydroxylase from rats pretreated with 3-methylcholanthrene still responds to acetone. We have reinvestigated the effects of pretreating rats with 3-methylcholanthrene upon the ability of the microsomal fraction to respond to acetone and other enhancing agents.

Methods

Male Wistar rats weighing 250 g, were pretreated with four, daily intraperitoneal injections of 3-methylcholanthrene 80 mg/kg, dissolved in corn oil. Control animals were treated with an equivalent volume of corn oil. Hepatic microsomes were prepared by the method of Ernster *et al.* [8] and suspended in 0·25 M sucrose, 0·05 M Tris pH 7·4. The drug metabolising activity of the freshly prepared microsomal fraction was determined over 30 min at 37° using a supporting system utilizing glucose-6-phosphate dehydrogenase as described by Mazel [9]. The formation of *p*-aminophenol from aniline was measured by the method of Schenkman *et al.* [10] and formaldehyde from aminopyrine by the method of Nash [11]. The concentration of aniline in the reaction mixture was 2·5 mM and aminopyrine 5·0 mM. In studies to determine the kinetic constants of aniline hydroxylation aniline concentrations of 0·1, 0·2, 0·3, 0·5 and 1·0 mM were employed. The protein content of the microsomal fraction was determined by the method of Lowry *et al.* [12].

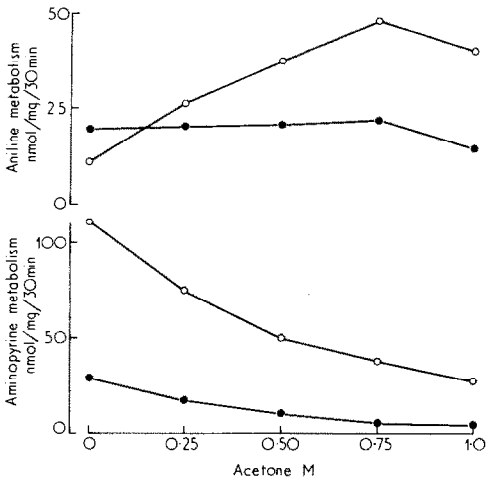

Fig. 1. The effect of acetone upon the metabolism of aniline and aminopyrine by the hepatic microsomal fraction from control (○) and 3-methylcholanthrene pretreated rats (●). Incubation conditions are described in the text. Acetone was added directly to the reaction mixture to give the concentrations shown.

Table 1. The effects of acetone on the kinetics of aniline hydroxylation by the microsomal fraction from control and 3-methylcholanthrene (3-MC) pretreated rats

	K_m (mM)	V_{max} (nmoles/mg per 30 min)
Untreated	0·234 ± 0·004	8·84 ± 0·51
Untreated + acetone	0·286 ± 0·016*	39·42 ± 1·89†
3-MC	0·266 ± 0·011	18·54 ± 0·38
3-MC + acetone	0·235 ± 0·045*	21·72 ± 0·89*

The incubation system employed is described in the text. The aniline concentrations were 0·1, 0·2, 0·3, 0·5 and 1·0 mM and the acetone concentration 0·62 M. The values represent mean ± S.E.M. of five determinations.
* P = <0·05, † P = <0·01 when compared to the appropriate control in the absence of acetone.

Results and discussion

Acetone added to the microsomal fraction from untreated rats produced a maximum enhancement of aniline hydroxylation of 436 per cent at 0·75 M, whilst aminopyrine demethylation was progressively inhibited up to 75 per cent at 1·0 M (Fig. 1). Pretreating rats with 3-methylcholanthrene itself produced a 77 per cent increase in aniline hydroxylation but almost completely abolished the ability of acetone to enhance aniline hydroxylation. Aminopyrine demethylation was inhibited 75 per cent by 3-methylcholanthrene pretreatment, although the remaining activity was inhibited by increasing concentrations of acetone in a manner analogous to the activity in the microsomal fraction from untreated animals. This may represent a non specific inhibition of the catalytic site by acetone.

The effect of pretreating rats with 3-methylcholanthrene upon the changes in the kinetics of aniline hydroxylation produced by acetone is shown in Table 1. Acetone added to the microsomal fraction from untreated animals produced only a 22 per cent increase in K_m but a 346 per cent increase in V_{max}. This differs from the findings of Anders [3] who reported an almost two fold increase in both the K_m and V_{max}. Pretreatment with 3-methylcholanthrene itself had little effect upon the K_m causing a 14 per cent increase ($P < 0·05$), but produced a 110 per cent increase ($P < 0·01$) in the V_{max}. Acetone added to the microsomal fraction from 3-methylcholanthrene pretreated animals now decreased the K_m by 12 per cent and increased the V_{max} by only 17 per cent.

Table 2. The effect of pretreating rats with 3-methylcholanthrene (3-MC) upon the enhancement of aniline hydroxylation by different agents

	Aniline hydroxylation (%)	
	Untreated	3-MC
Control	100·0 ± 2·9	100·0 ± 3·5
Acetone 0·75 M	436·0 ± 3·1	113·9 ± 1·9*
2,2′-bipyridyl 2·5 mM	345·4 ± 1·8	142·8 ± 3·1*
Metyrapone 1 mM	133·0 ± 1·8	82·3 ± 1·2*
Paraoxon 5 mM	170·6 ± 17·3	95·0 ± 5·0*
1,10-phenanthroline 4 mM	178·5 ± 1·4	57·0 ± 0·2*

The incubation system employed is described in the text. The concentration of an enhancing agent employed was that found to produce the maximal increase in aniline hydroxylation by the microsomal fraction from untreated rats. The results are expressed as per cent of the appropriate untreated or 3-MC treated control, and represent the mean ± S.E.M. of four determinations.
* P = <0·01 compared to the effect of the agent on the microsomal fraction from untreated rats.

The induction of hepatic microsomal mixed function oxidase activity by polycyclic hydrocarbons results in the formation of an aberrant form of cytochrome P-450, called cytochrome P-448, which lacks the type I binding site [6, 13]. The type II binding site is however unaffected. Chaplin and Mannering [5] have suggested that the binding of type I substrates to cytochrome P-450 results in a conformational change facilitating the flow of electrons within the substrate-haemoprotein complex. An alternative suggestion by Hayes and Campbell [14] is that binding to the type I site increases the availability of the substrate for the catalytic site. In either case an increase in the binding of aniline to the type I site, as produced by acetone would be expected to increase the rate of aniline hydroxylation. Destruction of the type I binding site *in vitro* by digesting the microsomal fraction with phospholipase C abolishes acetone enhancement [4]. The present work shows that an *in vivo* reduction in type I binding caused by 3-methylcholanthrene pretreatment also abolishes acetone enhancement.

The effects of pretreating rats with 3-methylcholanthrene upon the increase in aniline hydroxylation produced by other enhancing agents are shown in Table 2. The agents studied were 2,2′-bipyridyl [15], metyrapone [16], paraoxon [17] and 1,10-phenanthroline [15]. Pretreating rats with 3-methylcholanthrene markedly reduced the enhancing effects of all the agents studied and in some cases led to an inhibition of aniline hydroxylation. Leibman and Oritz [18] have previously reported that pretreatment with 3-methylcholanthrene reverses the enhancing action of metyrapone and Anders [7] has reported that 3-methylcholanthrene pretreatment reverses the enhancing effects of 2,2′-bipyridyl. It has so far proved difficult to ascribe a common mechanism of action to the various enhancing agents [1, 2]. The present results suggest however that all the enhancing agents studied may require an intact type I binding site to produce their effect.

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The influence of 6-hydroxydopamine on mouse brain acetylcholinesterase and glutamic acid decarboxylase activity

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Classical approaches to the study of function in the nervous system include biochemical inactivation and anatomical lesioning. The finding that 6-OHDA destroys catecholamine containing neurones has therefore been the subject of many studies. In adult animals the catecholamine containing nerve endings destroyed by 6-OHDA show no tendency for regeneration [1]. The intraventricular (i.v.) administration of 6-OHDA is initially associated with various behavioural changes, yet within days it is difficult to distinguish the experimental animals from the controls [2]. It seems possible that the residual catecholamine containing neurones, or other neural systems, are taking over the function of the destroyed neurones. The possibility of an influence of 6-OHDA on brain AChE (Acetyl-CoA: choline *O*-acetyltransferase E.C.3.1.1.7.) and GAD (L-glutamate 1-carboxylyase E.C.4.1.1.15) activity was examined since these enzymes are concentrated in nerve endings and play key roles in other putative transmitter systems.

Materials and methods

The i.v. injection technique of Brittain and Handley [3] was used to inject 200 μg of 6-OHDA (Labkemi, Goteberg, Sweden), made up in 0·1% (w/v) ascorbic acid in 5 μl. Female mice of the LAC/G strain, of approx. 25 g wt were used. The control animals received an equivalent volume of 0·1% ascorbic acid. All injections were administered 3 days prior to biochemical assay. One half of a mouse brain was used to determine GAD activity according to Lowe *et al.* [4], and the other half of the brain was used to assay AChE according to Ellman *et al.* [5]. Protein was assayed by the method of Lowry *et al.* [6].

Three mouse brains, frozen in liquid nitrogen, were pooled and used for the determination of the biogenic amines, NA, DA and 5HT, which were measured according to Brownlee and Spriggs [7].

Results and discussion

Following the injection of 6-OHDA the behaviour of the mice alternated between short periods of hyperexcitability and longer periods of sedation. About one mouse in eight entered into convulsions and died. A report has appeared describing the effects of 6-OHDA in the mouse [8]. These authors reported that doses in excess of 50 μg produced a high mortality rate. In our experiments the dose of 200 μg killed 12–15 per cent of the mice and a further 10 per cent showed varying degrees of ataxia. After three days the behaviour of the remainder of the mice was almost indistinguishable from saline-injected controls and these mice were used for the biochemical assays.

The i.v. injection of 6-OHDA 3 days prior to assay resulted in a statistically significant decrease in whole brain DA ($P < 0.001$) and NA ($P < 0.001$), although no significant change in the level of 5-HT was produced (Table 1). This decrease in brain catecholamines was associated with

Abbreviations used: 6-OHDA (6-hydroxydopamine), GAD (L-glutamic acid 1-carboxylyase E.C.4.1.1.15), ChaC (Acetyl-CoA: choline *O*-acetyltransferase E.C.2.3.1.6), DA (dopamine), NA (noradrenaline), 5-HT (5-hydroxytryptamine).