Br. J. Pharmac. (1978), **64**, 63–69.

THE DEVELOPMENT OF TOLERANCE TO KETAMINE IN RATS AND THE SIGNIFICANCE OF HEPATIC METABOLISM

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1 A decrease in sleeping time in rats pretreated with ten daily doses of ketamine compared to controls is shown.

2 This decrease in sleeping time is associated with a more rapid decrease in circulating and brain levels of ketamine and its *N*-demethylated metabolite and higher levels of the subsequent oxidation metabolite in the pretreated animals.

3 Metabolism of ketamine to its *N*-demethylated metabolite by liver homogenates *in vitro* was more rapid when the livers were obtained from ketamine pretreated rats.

4 Microsomal preparations from rat liver were capable of metabolizing ketamine to its *N*-demethylated metabolite and this metabolite to the subsequent oxidation metabolite *in vitro*. The V_{max} and K_m for the first reaction calculated from loss of substrate were 433 mol mg^{-1} protein h^{-1} and 0.133 mM respectively and 199 nmol mg^{-1} protein h^{-1} and 0.121 mM for the second reaction.

5 The results indicate that tolerance to ketamine in rats is associated with increased hepatic metabolism which can also be demonstrated *in vitro* in liver homogenates.

Introduction

Ketamine is often used in the anaesthesia of children (Roberts, 1967) particularly when repeated anaesthesia is required such as for the dressing of burns (Roberts, 1967; Corssen & Oget, 1971) and the radiotherapy of tumours (Cronin, Bonsfield, Hewett, McLellan & Boulton, 1972; Bennett & Bullimore, 1973). The possibility that tolerance might develop to repeated doses of the drug was suggested by the work of Bree, Feller & Corssen (1967) in monkeys and clinically by Bjarnsen & Corssen (1967) in patients given ketamine on more than eight occasions and by Bennett & Bullimore (1973) in seven out of ten children given ketamine repeatedly over several weeks. Cronin *et al.* (1972) also recorded the need to increase the dose of ketamine in order to produce adequate sedation in a 6 month old baby after the sixth dose of the drug. Experimentally, Douglas & Dagirmanjian (1975) have noted the development of tolerance to ketamine in young, adult male rats after the third dose.

It has been suggested (Cohen & Trevor, 1974) that metabolic inactivation of ketamine was unlikely to be responsible for the termination of its hypnotic effects, and that redistribution plays a major part in the cessation of its actions. However, Piel, Aldrete & Jones (1969) found that phenobarbitone pretreatment shortened the duration of action of ketamine indicating a role for hepatic metabolism.

It was considered that an investigation of the development of tolerance to ketamine coupled with an examination of its metabolism under these circumstances may provide some information on the significance of metabolism in the termination of the effects of this drug. Preliminary reports of part of these studies have previously been published (Livingston & Waterman, 1976; 1977).

Methods

The rats used in this study were albino Wistar rats from a cross-fostered inbred colony. They were fed a standard diet and water *ad lib.* and housed under constant conditions of temperature and light.

In vivo *studies*

Groups of 24 male rats, 75 g body weight, were injected intraperitoneally daily for 10 days before experimentation. The control group received an injection of 0.9% w/v NaCl solution (saline) whilst the treated group received an injection of ketamine ('Vetalar', Parke-Davies Ltd.) at a dose rate of 40 mg/kg. On the eleventh day both groups received a single intraperitoneal injection of ketamine (75 mg/kg). Four rats from each group were then killed (by decapitation)

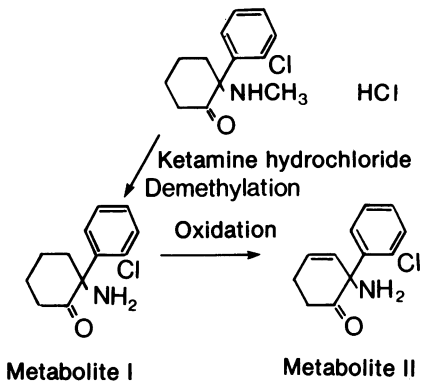

Figure 1 The structure of ketamine, its demethylated metabolite (metabolite I) and the subsequent oxidation product (metabolite II).

at 5, 10, 20, 30, 45 and 60 min after injection. Heparinised mixed arterial and venous blood samples were collected and the brains were dissected out. The time to onset of the loss of the righting reflex (onset time) and the time taken to regain the righting reflex (sleeping time) were measured in the 45 and 60 min groups. Plasma was collected by centrifugation of the blood at 1000 *g* for 10 min. The brains were homogenized in isotonic saline to give a 10% homogenate, which was centrifuged at 1500 *g* for 30 minutes in a refrigerated centrifuge. The supernatant was then collected and together with the plasma stored frozen until assay. There were no significant levels of ketamine or metabolites present in the pellet.

The concentrations of ketamine, its *N*-demethylated metabolite (metabolite I) and the subsequent oxidation product (metabolite II), (Figure 1) in the samples were assayed by a gas liquid chromatographic (g.l.c.) method based on that of Chang & Glazko (1972). The samples were assayed against known amounts of ketamine and its two metabolites, with 2-amino-2-(*o*-bromophenyl)-2-methylamino cyclohexanone used as the internal standard. The method differed from that of Chang & Glazko (1972) only in one respect, that nitrogen was used as the carrier gas rather than a methane/argon mixture.

In vitro *studies*

Metabolism of ketamine by liver homogenates. Six week old male rats were pretreated with ketamine or saline for a 10 day period as previously described. The rats were decapitated and the livers rapidly removed and placed on ice. They were cleaned of blood and a 10% homogenate in isotonic saline was prepared; 0.5 ml aliquots were placed in conical flasks containing the following final concentrations: 18 mM Na phosphate buffer, pH 7.4, 0.08 mM NADP, 50 mM nicotinamide, 6 mM $MgCl_2$ and 6 mM glucose-6-phosphate. The flasks were placed in a shaking incubator at 37°C and preincubated for 15 min whilst being aerated. The reaction was then initiated by addition of 0.25 ml ketamine (1 mg/ml), giving a final volume of 2.5 ml with an initial concentration of ketamine of 100 μg/ml; 0.1 ml samples were taken at 0, 15, 30, 60 and 120 min incubation and added to 0.9 ml 0.1 M HCl. These samples were extracted and assayed as described previously.

Metabolism of ketamine and metabolite I by liver microsomal preparations. Livers from adult male rats (250 to 300 g) were excised after decapitation and 10% homogenates in 0.32 M sucrose were prepared. These homogenates were centrifuged at 1000 *g* for 5 min, the resulting supernatant was recentrifuged at 10,000 *g* for 20 min and the supernatant retained. This supernatant was centrifuged at 70,000 *g* for 30 min to prepare microsomal pellets. These pellets were resuspended in 0.32 M sucrose to give a final protein concentration of between 1 and 3 mg/ml as determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Aliquots (0.8 ml) of the microsomal suspension were added to reaction vessels containing the following final concentrations: 11 mM Na phosphate buffer pH 7.4, 5 mM $MgCl_2$ and 6 mM glucose-6-phosphate, and 1 unit glucose-6-phosphate dehydrogenase. The vessels were allowed to equilibrate for 15 min at 37°C whilst aerated. NADP was added to a final concentration of 0.08 mM and the reaction initiated by the addition of either 0.1 ml ketamine at concentrations varying from 0.37×10^{-4} to 4.4×10^{-4} M or metabolite I at concentrations of 0.2×10^{-4} to 4×10^{-4} M, to give a final reaction volume of 2.5 ml. Samples (0.1 ml) were then taken after 20 min and extracted for assay as previously described; 0.1 ml samples were also taken for protein determination. The results were expressed in terms of nmol of metabolite produced per mg protein per h (nmol mg^{-1} protein h^{-1}), and plotted as a double reciprocal plot against the initial concentration of the substrate.

Results

In vivo *metabolism*

Pretreatment of rats with 10 daily doses of ketamine resulted in a significant ($P < 0.001$) reduction in the sleeping time compared to the control animals. Sleeping time in the ketamine pretreated groups was 11.73 ± 0.95 min, (mean ± s.e. mean of 8 rats) whilst in the saline pretreated groups the sleeping time was 23.11 ± 0.95 min. The time taken to lose the righting

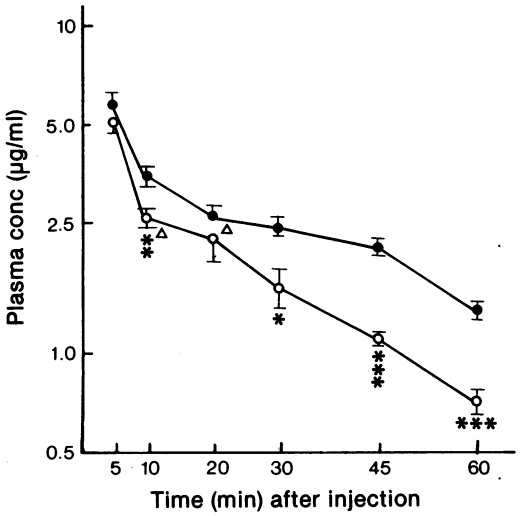

Figure 2 Plasma levels of ketamine after intraperitoneal administration of ketamine (75 mg/kg) to saline (●) or ketamine (○) pretreated rats; (△) indicates the mean time at which rats in each group regained their righting reflex. Values are mean results from 4 animals; vertical lines show s.e. means. The levels of significance refer to the difference from the controls at the same time point: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

reflex (onset time) was longer in the pretreated rats, 2.18 ± 0.37 min as against 1.75 ± 0.11 min but this difference was not statistically significant.

Plasma levels. The levels of ketamine in the plasma obtained from the groups of rats killed at various times after the injection are shown in Figure 2. It can be seen that the difference in levels between the ketamine pretreated rats and the control group was not significant at 5 min, but this difference was significant at all the subsequent time points. The plasma half-life for the initial rapid phase of decline in the levels was 7 min for the control animals and 5 min for the pretreated group. It is also interesting to note that recovery occurred at almost exactly the same plasma ketamine level in each group, at approximately 2.5 μg ketamine/ml plasma.

The comparison of the metabolite I levels in the plasma is shown in Figure 3. The pattern of decline is almost identical in the two groups but the levels of metabolite I in the pretreated group are consistently below those of the control group with the differences being significant at 10 and 45 min ($P < 0.05$).

The pattern of plasma levels of metabolite II, however, was very different from those of metabolite I as shown in Figure 4. In the control animals the levels were low at 5 min but rose rapidly to a peak 30

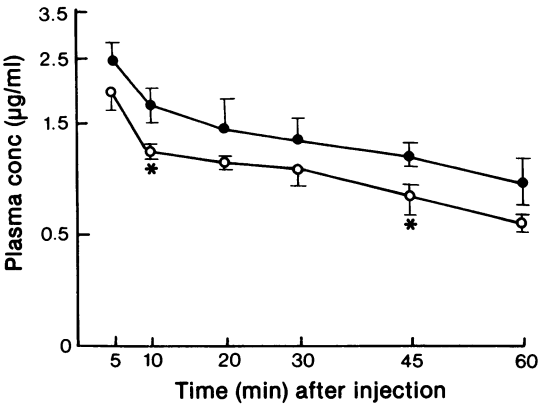

Figure 3 Plasma levels of metabolite I after intraperitoneal administration of ketamine (75 mg/kg) to saline (●) or ketamine (○) pretreated rats. Values are mean results from 4 animals; vertical lines show s.e. means. The levels of significance refer to the difference from the controls at the same time point: $^{*}P < 0.05$.

min after injection after which time they declined slowly. The plasma levels in the pretreated rats were three times the control level at 5 min and then rose more rapidly to reach a peak at 20 min. The metabolite II levels were significantly higher in the pretreated animals at 5, 10, 20 and 60 min after injection of ketamine.

Brain levels. The values obtained from the rat brain homogenates for ketamine and its metabolites are shown in Figures 5 and 6. It can be seen that brain ketamine levels were high in both groups of rats at 5 min but that they declined rapidly over the next 15 min. This decline was more rapid in the ketamine pretreated rats with a half life of approximately 5 min compared to a value of 10 min in the control animals. The levels in the brains of the pretreated animals continued to be lower than those in the control animals over the next period of decline up to 60 min and the differences between the levels were significant at 10, 30, 45 and 60 min. It can also be seen that the brain levels at recovery of righting reflex were similar in both groups of rats (25–28 μg/g).

The pattern of distribution of the two major metabolites was similar to that seen in the plasma. The metabolite I levels showed a more rapid decrease in the ketamine pretreated animals and the brain levels of metabolite I in the pretreated group were significantly lower at 30, 45 and 60 min. The metabolite II levels were not detectable in the pretreated group until 10 min after injection and until 20 min after injection in the control animals. The levels in the pretreated group seem to have levelled off by 45 min

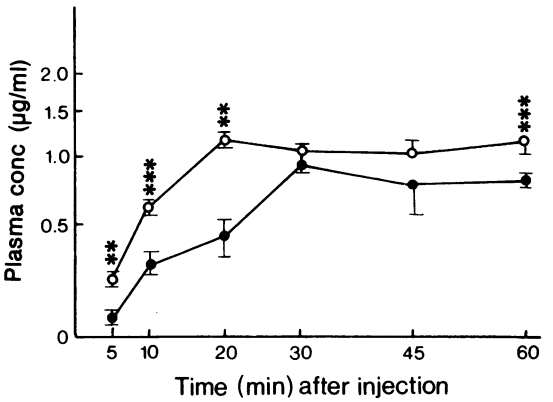

Figure 4 Plasma levels of metabolite II after intraperitoneal administration of ketamine (75 mg/kg) to saline (●) or ketamine (○) pretreated rats. Values are mean results from 4 animals; vertical lines show s.e. means. The levels of significance refer to the difference from the controls at the same time point: $^{**}P < 0.01$; $^{***}P < 0.001$.

but the levels in the control group were still rising slowly at 60 min and had almost reached the levels of the pretreated group. The brain levels of metabolite II in the pretreated rats were significantly higher than those in the control group at 10, 20 and 45 min.

In vitro *metabolism*

Preliminary experiments had indicated that *in vitro* the rate of metabolism of ketamine by homogenates of brain, lung and kidney was very slow, but that appreciable metabolism of ketamine to metabolite I could take place with liver homogenates.

The incubation of ketamine with homogenates of liver from saline-treated control animals or ketamine pretreated rats resulted in the decrease in the concentration of ketamine and the increase in the concentration of metabolite I in the medium, as shown in Figure 7. It can be seen that the livers from ketamine pretreated rats metabolized ketamine faster than those of control rats, with ketamine levels being significantly lower after 30, 60 and 120 min, whilst the concentration of metabolite I was significantly higher after 10, 15, 30, 60 and 120 min incubation. The incubations showed that there was an initial fairly rapid decrease in ketamine levels but that the metabolism in the second hour was relatively much lower than that in the first hour. The rates of metabolism of ketamine for the pretreated and control rats were significantly different at the level $P < 0.01$ when the 0 to 15 and 0 to 30 min periods were considered but after 120 min there was no significant difference in the rates for the two groups.

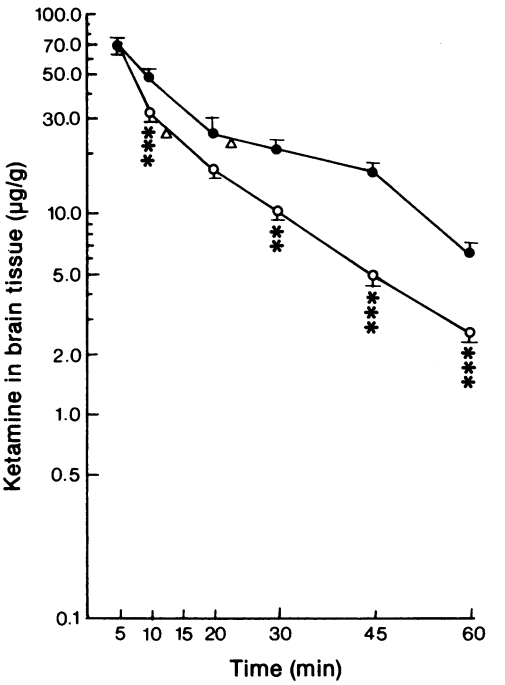

Figure 5 Brain levels of ketamine after intraperitoneal administration of ketamine (75 mg/kg) to saline (●), or ketamine (○) pretreated rats at various times after injection; (△) indicates the mean time at which rats in each group regained their righting reflex. Values are mean results from 4 animals; vertical lines show s.e. means. Significantly different from control: $^{**}P < 0.01$; $^{***}P < 0.001$.

Kinetic studies. The studies with different concentrations of ketamine were performed using a different incubation mixture since the studies described above failed to show any metabolism of metabolite I to metabolite II. The addition of exogenous glucose-6-phosphate dehydrogenase to the medium was found to result in the production of both metabolites by the liver homogenates and also by the liver microsome preparations. With microsomes a range of substrate concentrations was used and this allowed the construction of Lineweaver-Burk double reciprocal plot (1934) using regression analysis for the conversion of metabolite I to metabolite II (Figure 8). From this plot it was found that maximal velocity (V_{max}) and the Michaelis constant (K_m) for the conversion of metabolite I to metabolite II were 199 nmol mg^{-1} protein h^{-1} and 0.121 mM respectively. Calculations based on the rate of disappearance of ketamine from the medium gave V_{max} of 433 nmol mg^{-1} protein h^{-1} and a K_m of 0.133 mM for the reaction of ketamine to metabolite I.

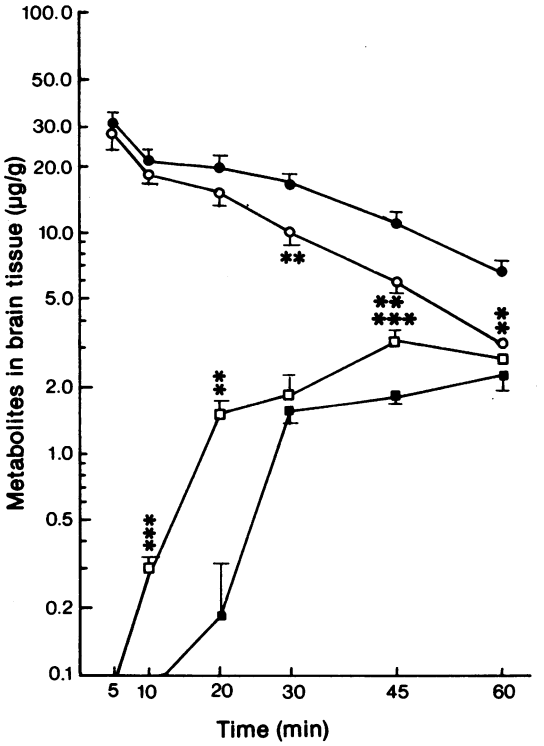

Figure 6 Brain concentrations of metabolites I and II after intraperitoneal administration of ketamine pretreated rats: (●) metabolite I levels in saline pretreated rats; (○) metabolite I levels in ketamine pretreated rats; (■) metabolite II levels in saline pretreated rats; (□) metabolite II levels in ketamine pretreated rats. Values are mean results from 4 animals; vertical lines show s.e. means. Significantly different from control: $^{**}P < 0.01$; $^{***}P < 0.001$.

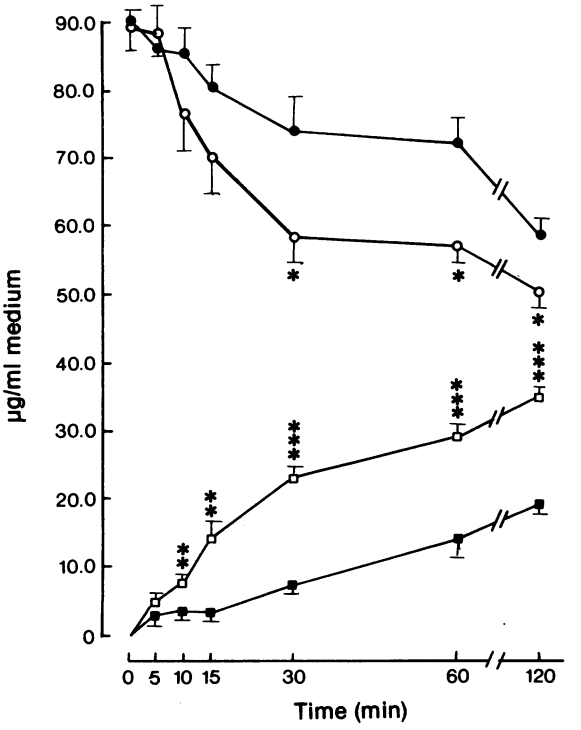

Figure 7 Concentration of ketamine and metabolite I in μg/ml of incubation medium containing homogenates of rat liver against incubation time: (●) ketamine concentrations from control rat livers; (○) ketamine concentrations from pretreated rat livers; (■) metabolite I concentrations from control rat livers; (□) metabolite I concentrations from pretreated rat livers. Values are mean of 6 experiments; vertical lines show s.e. means. Significance of difference from control at same time point: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Discussion

Reports of the induction of tolerance following repeated doses of ketamine in clinical cases prompted experimental studies on this problem. The work of Douglas & Dagirmanjian (1975) suggested that significant tolerance could be induced in rats by the third successive dose given at four day intervals. Preliminary studies (Livingston & Waterman, 1976) indicated that, in our hands, this dose regime did not produce reliable reductions in sleeping times even after five doses, but a pretreatment regime similar to that used by Bree *et al.* (1967) in monkeys, namely ten daily doses, reliably produced in the rats, as it did in the monkeys, a significant decrease in the sleeping time when compared to control animals, and this 10 daily dose pretreatment schedule resembles the clinical pattern of usage of ketamine after which tolerance seems to develop (Bjarnsen & Corssen, 1967).

Ketamine is metabolized by the liver (Chang, Dill & Glazko, 1965) and Piel *et al.* (1969) found that pretreatment with phenobarbitone resulted in a significant decrease in sleeping time following ketamine administration and that pretreatment with SKF-525A, a potent inhibitor of microsomal enzyme systems, resulted in a prolongation of ketamine anaesthesia. However, Cohen & Trevor (1974) found that when they pretreated with these two drugs they could not alter sleeping times significantly although they could affect the rate of metabolism of the drug *in vitro*.

In this study, enzyme induction by pretreatment with ketamine was seen to occur *in vivo*, indicated

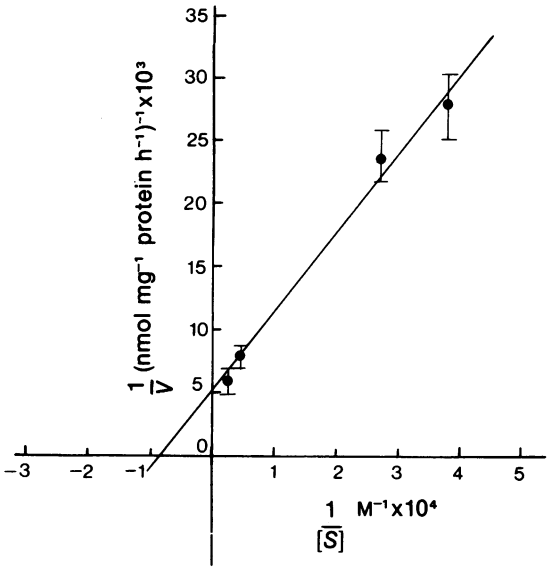

Figure 8 Lineweaver-Burk plot of oxidation of metabolite I to metabolite II. Rate of conversion ($1/V$) plotted against substrate concentration ($1/[S]$). Values are mean results of 4 experiments; vertical lines show s.e. means. Line plotted by regression analysis. V_{max} = 199 nmol mg^{-1} protein h^{-1}; K_m = 0.121 mM.

by a more rapid fall in plasma and brain levels of ketamine and metabolite I and a rise in metabolite II concentrations in the pretreated rats compared to the controls. These effects were associated with a significant decrease in the sleeping time in the pretreated animals.

The *in vivo* findings were confirmed by the demonstration of an increased rate of metabolism of ketamine by liver homogenates *in vitro* when the livers were taken from rats pretreated with ketamine. These findings therefore suggest that enzyme induction is mainly responsible for the development of tolerance to ketamine. These findings are not in agreement with those of Chang & Glazko (1974) who were unable to enhance the *N*-demethylation activity of microsomal enzymes by ketamine pretreatment but are indirectly supported by the studies of Cohen & Trevor (1974) and Douglas & Dagirmanjian (1975). It is possible that the discrepancy between the work of Cohen & Trevor (1974) and Piel *et al.* (1969) on the influence of phenobarbitone and SKF 525A on the actions of ketamine may be explained by the different routes of ketamine administration used by these two groups.

The *N*-demethylated metabolite (metabolite I) was produced rapidly and accumulated in the brain with a brain:plasma ratio at recovery of approximately 10:1, similar to that seen with ketamine. The detection of the cyclohexanone oxidation product (metabolite II) in plasma and brain was in contrast to the findings of Cohen, Chan, Way & Trevor (1973) and Cohen & Trevor (1974) who were unable to demonstrate this metabolite *in vivo* or *in vitro* in their rats. The levels of metabolite II were much lower than metabolite I in the brain and were not detectable for the first 10 min after injection and initial plasma levels were also low. These differences in results may have several explanations. There could be strain differences in the rats, or the differences in experimental design could be the reason. Cohen and co-workers assayed plasma and brain tissue following a much lower dose of ketamine (20 mg/kg i.v.) and collected samples only up to 10 min after administration. It is possible the relatively low levels of metabolite present in the first 10 min after ketamine administration and the low dose rate used might account for their failure to detect it. This would be supported by the finding of White, Johnston & Pudwill (1975) who isolated metabolite II from the plasma and brain tissue of rats after doses of ketamine in excess of 20 mg/kg.

The rate of metabolism of ketamine by lung and kidney tissue was very slow compared to liver homogenates and it would appear that, in the rat at least, extra-hepatic metabolism does not play a significant role in the degradation of this drug. The failure of brain tissue to metabolize ketamine indicates that the cerebral accumulation of metabolite I must be the result of extra-cerebral degradation as suggested by Cohen *et al.* (1973). Cohen & Trevor (1974) have previously shown that pretreatment with phenobarbitone increases the ability of liver enzymes to metabolize ketamine *in vitro* and we have shown that pretreatment with ketamine itself can increase the rate of metabolism of ketamine (*in vitro*) by 122% overall. Thus the ability to increase metabolism *in vitro* by pretreatment confirms the results *in vivo*, and it would appear that the decrease in the plasma half-life of the drug noted *in vivo* was caused by an increased rate of hepatic metabolism.

These studies on the metabolism of ketamine (*in vitro*) by liver homogenates were done with a medium based on that described by Cohen & Trevor (1974) and the only detectable metabolic product of ketamine was metabolite I, which was not metabolized further; only when excess glucose-6-phosphate dehydrogenase was added was metabolite II produced. The studies with a liver microsomal preparation were carried out using this incubation medium which contained exogenous glucose-6-phosphate dehydrogenase and it was found that with this medium conversion of metabolite I to metabolite II was achieved by the microsomes. This additional metabolism in the presence of added glucose-6-phosphate dehydrogenase

may indicate that there is insufficient endogenous enzyme available for the reaction metabolite I → II to proceed following the preparation procedure for homogenates and microsomes.

Calculations involving the amounts of metabolites produced and examination of the g.l.c. traces indicated that neither significant alternative metabolism of ketamine nor further metabolism of metabolite II had occurred.

Studies on the kinetics of the enzymic conversions of ketamine to metabolite I and of metabolite I to metabolite II indicated that the initial reaction in the conversion of the parent compound had a maximum rate far in excess of that of the subsequent step, however, the Michaelis constants for the two reactions were similar. Some recent studies (White, Marietta, Pudwill, Way & Trevor, 1976) on the effects of halothane on ketamine anaesthesia yielded values for K_m and V_{max} for the enzymatic degradation of ketamine which are very close to those reported in this study.

The development of tolerance to ketamine has been shown to occur in rats after pretreatment with the drug and this had been shown to be associated with the induction of liver microsomal enzyme systems which was demonstrated *in vitro*; however, the possibility that cellular tolerance may also develop cannot be eliminated.

We are grateful to Dr T. Chang of Parke Davis & Co. Ltd. for the gift of standards of ketamine and its metabolites and of the internal standard used in the assays. A.E.W. is a Wellcome Research Training Scholar.

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(*Received January 27, 1978. Revised April 20, 1978.*)