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Enhanced development of tolerance to pentobarbital by desipramine inhibition of pentobarbital metabolism*

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It is known that the duration and intensity of pentobarbital (PB) action in the rat, rabbit, dog and man are determined by the rate at which the drug is metabolized by the liver [1–4]. The development of tolerance to PB is a well-established phenomenon resulting from increased metabolism of the drug due to hepatic enzyme induction [5–8]. The degree of acute tolerance to PB has been found to correlate positively with the size of the tolerance-inducing dose of the barbiturate [9]. It seemed possible that the treatment with a combination of desipramine (DMI) or another related tricyclic antidepressant and PB might potentiate the development of tolerance to PB, since Liu *et al.* [10] have reported recently that acute treatment with a tricyclic antidepressant prolongs the hypnotic action of PB in the rat through inhibition of PB metabolism. The combination of a tricyclic antidepressant and PB may act in a manner similar to a larger dose of PB alone, thus promoting induction of the microsomal enzyme systems.

Since long-term administration of tricyclic antidepressants is required for therapy of depressed patients and many patients with mental disorders receive combinations of antipsychotic drugs and other drugs including hypnotics [11], the present study was designed to investigate the effect of tricyclic antidepressants on the development of tolerance to PB, using DMI as a model of a drug which interacts with PB.

Male albino rats of the Charles River CD strain (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 150–200 g were used. Purina laboratory chow and water were fed *ad lib.* Prior to the acute phase experiment, rats were fasted for 16 hr with free access to water. DMI hydrochloride was dissolved in distilled water and diluted with an equal volume of 1.8% sodium chloride solution. [^{14}C]-2-pentobarbital sodium ([^{14}C]-PB) was purchased from ICN Chemical and Radioisotope Division (Irvine, Calif.). The radiochemical purity of the [^{14}C]-PB was 98 per cent when checked by thin-layer chromatography. PB sodium was mixed with [^{14}C]-PB in 0.9 per cent saline to yield a solution with a specific activity of 15 μCi/25 mg. Drug solutions were administered intraperitoneally at a volume of 2.0 ml/kg. The control animals were injected with an equivalent volume of saline. Doses of the drug are expressed hereafter in terms of the salts.

Sleeping time was taken as the interval between the loss and regaining of the righting reflex. Rats were kept warm while sleeping by placing them on a table under incandescent lamps (100 watts). The sleeping time studies were always started between 10:00 and 11:00 a.m. to avoid effects due to circadian rhythm variation in PB response [12, 13]. The development of tolerance to PB was assessed by comparing the sleeping times of drug-treated and saline-treated rats receiving the same challenge dose of PB and by determining the hepatic microsomal activity of PB hydroxylase. The criterion of tolerance to PB was either a significant reduction of sleeping time or a significant increase in enzyme activity.

For study of the effect of DMI on concentrations of unchanged PB and its metabolites in tissues on awakening, DMI (25 mg/kg) was given 1 hr before administration of [^{14}C]-PB (25 mg/kg). Control rats received saline and the same dose of [^{14}C]-PB. Rats were killed by decapitation immediately upon awakening. Blood was collected in heparinized tubes and samples of plasma were removed immediately after centrifugation of blood. The whole brain, liver and kidneys were removed and frozen at $-20°$ until further analyses.

Two experiments were performed to assess the influence of DMI on the development of tolerance to PB.

Experiment No. 1. To study the effect of a single dose of DMI on the development of tolerance to PB, rats were given DMI (25 mg/kg) or saline 1 hr before receiving a 40 mg/kg dose of PB on day 1. On days 2 and 3 all rats were challenged again with 40 mg/kg of PB. In order to measure the pentobarbital hydroxylase activity in liver microsomes, the rats were killed on day 4. Rats receiving only saline injections for 3 days were used as controls for comparison of enzyme activity.

Experiment No. 2. Rats were divided randomly into four equal groups: (1) saline control; (2) DMI group; (3) PB group; and (4) DMI and PB group. From day 1 through day 5, group 1 rats received two injections of saline; group 2 received DMI (15 mg/kg) and saline; group 3 received saline and PB (25 mg/kg) and group 4 received the same doses of DMI and PB. The two injections were given at an interval of 1 hr. Day 6 was used as a rest period to permit the excretion of residual amounts of drugs. On day 7, one-half of the rats in each group were challenged with 40 mg/kg of PB, the other half were sacrificed and the activity of pentobarbital hydroxylase in liver microsomes was determined.

The preparation of liver microsomes and the assay conditions for PB hydroxylation were similar to those described by Liu *et al.* [14]. The activity of PB hydroxylase was measured by the formation of total PB metabolites as described by Kuntzman *et al.* [15]. Microsomal protein concentration was determined by the method of Lowry *et al.* [16]. For measurement of radioactivity, whole brain, liver and kidneys were homogenized as 20% (w/v) suspensions in distilled water with the addition of a few drops of isoamyl alcohol. Two-ml aliquots of plasma, tissue homogenate or microsomal incubation mixture were used for separation of unmetabolized PB and total PB metabolites, according to the extraction method of Kuntzman *et al.* [15]. A 2.0-ml aliquot of each organic phase was counted in 15 ml of a scintillation fluid consisting of 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) dissolved in 1 liter toluene and 0.5 liter Triton X-100. Radioactivity was counted in a Packard Tri-Carb liquid scintillation counter, model 3330 (Packard Instrument Co., Inc., Downers Grove, Ill.). Samples were corrected for quenching with [^{14}C]-toluene as an internal standard.

Sleeping time of rats (96.2 $\pm$ 9.15 min, mean $\pm$ S. E.) treated with DMI (25 mg/kg) 1 hr before administration

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Table 1. Effect of DMI on the tissue distribution of unchanged PB and its metabolites on awakening*

Tissue	Unchanged PB	Metabolites
Liver		
Control	146 ± 2.3	58.4 ± 4.1
DMI	153 ± 12.1	38.5 ± 2.8†
Kidney		
Control	93.4 ± 3.1	88.2 ± 6.1
DMI	96.2 ± 7.3	68.1 ± 8.6‡
Plasma		
Control	70.2 ± 3.4	41.3 ± 2.4
DMI	67.1 ± 3.8	26.4 ± 1.4†
Brain		
Control	87.2 ± 2.1	6.20 ± 0.28
DMI	85.5 ± 5.6	6.40 ± 0.93

* Rats were injected i.p. with saline or DMI (25 mg/kg) 1 hr before i.p. administration of [^{14}C]-PB (25 mg/kg, 15 μCi/kg) and killed by decapitation at times of awakening. Each figure represents the mean ± S. E. M. of six rats, and is expressed as nmoles of PB or its equivalent/g of wet tissue or ml of plasma.

† $P < 0.01$.

‡ $P < 0.05$.

of PB (25 mg/kg) was more than double that in control rats (42.8 ± 8.32 min). DMI alone induced no sleep in any of the rats tested. Table 1 presents the concentration of unchanged PB and its metabolites in several organs measured at the time of awakening. The concentration of unchanged PB in liver, kidney, plasma and brain did not differ significantly between DMI-treated and control rats. However, all DMI-treated rats has significantly lower concentrations of PB metabolites in liver, kidney and plasma. The brain/plasma concentration ratio of unchanged PB in DMI-treated rats (1.34 ± 0.028) was not significantly different from that in controls (1.32 ± 0.047) at the time of awakening. These results thus confirm our previous suggestion of an inhibition of PB metabolism by DMI [10] and substantiates the evidence that central nervous system sensitivity to the hypnotic effect of PB is not changed by DMI treatment.

The results shown in Fig. 1 indicate that DMI significantly prolonged PB sleeping time even when given as a 24-hr pretreatment; however, the potentiating effect was much less than that observed in the 1-hr pretreatment experiment. This finding suggests that DMI has a long half-life in rats, which agrees well with the finding of Dingell *et al.* [17]. Since DMI given as a 48-hr pretreatment no longer produced a significant effect on PB sleeping time, 48 hr was used as a rest period before measurement of sleeping time on DMI-treated rats in subacute experiments to permit the excretion of a residual amount of DMI. The fact that DMI given as either a 48- or 72-hr pretreatment produced no significant effect suggests that DMI does not exert a biphasic effect (inhibition and stimulation) on PB hypnotic action as a function of different pretreatment times.

Figure 2 shows tolerance developed to PB after a single dose of DMI followed by 2 days of daily administration of PB as compared to tolerance developed after 2 days of daily administration of PB alone. It is interesting to note that tolerance to PB was developed within 24 hr after a single dose of PB, as evidenced by a significantly decreased sleeping time on day 2 for the saline-treated group. This finding is in agreement with previous reports [5, 9]. The longer sleeping time in DMI-treated rats on day 2 was apparently due to the presence of a residual amount of DMI. When the inhibitory effect of DMI was no longer evident on day 3, enhanced development of tolerance to

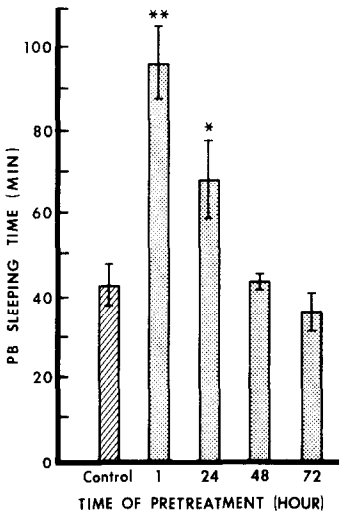

Fig. 1. Effect of pretreatment with DMI at different times on sleeping time of PB. Rats were pretreated with DMI (25 mg/kg, i.p.) and 1, 24, 48 or 72 hr later challenged with PB (25 mg/kg, i.p.) for measurement of sleeping time. Control rats received saline 1 hr prior to injection of PB. Each figure represents the mean ± S. E. M. of five rats. Single and double asterisks (*) and (**) indicate significant difference from control rats at $P < 0.05$ and $P < 0.01$ respectively.

PB by DMI was observed. This additional development of tolerance to PB stimulated by DMI was associated with a marked increase in the liver microsomal pentobarbital hydroxylase activity (1.35 ± 0.12 nmoles of PB metabolites

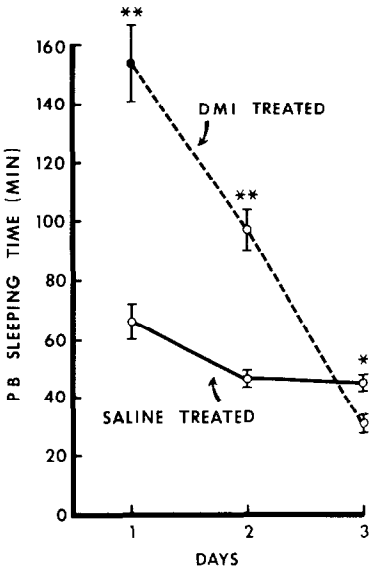

Fig. 2. Effect of a single dose of DMI on the development of tolerance to PB. Rats were pretreated with DMI (25 mg/kg i.p.) 1 hr before administration of PB (40 mg/kg, i.p.) on day 1. Saline-treated rats received saline and the same dose of PB. All rats received two additional doses of PB (40 mg/kg, i.p.) on days 2 and 3 for measurements of sleeping times. Each point represents the mean ± S.E.M. of five rats. Single and double asterisks (*) and (**) indicate significant difference from saline controls at $P < 0.05$ and $P < 0.01$ respectively.

Table 2. Effect of subacute administration of a combination of DMI and PB on body weight gained, PB sleeping time and PB hydroxylase activity*

Experimental group	Body weight gained (g)	PB sleeping time (min)	PB hydroxylase activity (nmoles metabolites/mg protein/min)
Saline	38 ± 3.2	72 ± 8.1	0.61 ± 0.04
DMI	26 ± 2.8†	73 ± 8.2	0.74 ± 0.06
PB	32 ± 1.5	55 ± 2.8‡	0.88 ± 0.05†
DMI + PB	25 ± 2.5‡	33 ± 2.0‡,§	1.17 ± 0.07‡,‖

* Rats were pretreated i.p. once daily for 5 days with saline, DMI (15 mg/kg), PB (25 mg/kg) or the same dose of DMI + PB at 1-hr intervals. One-half of the rats were challenged with PB (40 mg/kg) for measurement of sleeping time, and the other half were killed by decapitation for measurement of PB hydroxylase activity in the isolated liver microsomes 48 hr after the last dose of pretreatment.
†$P < 0.05$ compared to saline controls.
‡$P < 0.01$ compared to saline controls.
§$P < 0.05$ compared to PB-treated group.
‖$P < 0.01$ compared to PB-treated group.

formed/mg of microsomal protein/min) measured on day 4 which increased to 200 per cent of control value (0.68 ± 0.07) as compared to 150 per cent of control in rats receiving PB only (1.02 ± 0.05, $P < 0.01$, as compared to 1.35 ± 0.12).

The effect of repeated daily administration of DMI on the development of tolerance to PB is shown in Table 2. Daily treatment with DMI alone for 5 days produced no significant change in sleeping time to PB given 48 hr after the final dose of DMI. Repeated treatment with a combination of DMI and PB for 5 days produced a greater degree of tolerance to PB than repeated treatment with PB alone, as shown by the significant difference between the sleeping times of these two groups measured 48 hr after the last pretreatment. The enhanced development of tolerance to PB by subacute treatment with DMI and PB was also indicated by the fact that rats pretreated with DMI plus PB had substantially higher PB hydroxylase activity than rats receiving PB alone, as shown in Table 2. The same table also shows that rats pretreated with DMI alone or DMI plus PB gained less body weight at the end of the experiment than those rats pretreated with saline or PB alone. Wet liver weight and liver microsomal protein concentrations were not significantly different among the groups.

It has been well demonstrated by several investigators that tolerance, developed to PB after either single or repeated treatments, is due mainly to increased activity of hepatic microsomal drug-metabolizing systems, especially hydroxylating enzymes [5–8, 18]. The participation of liver enzymes in the development of tolerance to PB was confirmed by experiments in which tolerance did not develop within 48 hr in partially hepatectomized rats, while it was present in sham-operated rats [19], and by similar experiments employing pretreatment of the animals with carbon tetrachloride [20] or ethionine [8] which, by impairing the ability of the liver to produce enzymes, inhibited the development of acute tolerance to PB.

In view of the foregoing discussion, it is not surprising to note that concurrent treatment with a combination of DMI and PB stimulated development of tolerance toward PB, as evidenced by both the sleeping time data and by measures of PB hydroxylase activity. The results appear to be due solely to the ability of the PB to stimulate the enzymes, because administration of DMI alone neither decreased the PB sleeping time nor stimulated PB hydroxylase activity. DMI has been shown to inhibit the metabolism of PB both *in vivo* and *in vitro* [10]. Apparently, the combination of DMI and PB acts in a manner similar to a higher dose of PB alone. In this instance, PB would remain in its active (enzyme-stimulating) state for a longer period of time in the liver, thus allowing for a greater effect in promoting the induction of the hepatic enzyme systems. The enhanced development of tolerance to PB was observed in rats receiving a single dose of DMI and multiple doses of PB, and in animals receiving repeated daily treatments with DMI and PB for 5 days. In both experiments, the decreased sleeping time in DMI-treated rats was accompanied by a higher activity of PB hydroxylase. Similar results were found on the effects of ethanol on the metabolism and the development of tolerance to PB. Recently, Liu *et al.* [14] reported that the addition of ethanol *in vitro* inhibited the activity of liver microsomal PB hydroxylase and that ethanol given along with PB potentiated the increase in PB hydroxylase activity induced by PB. Enhanced development of tolerance to PB hypnosis was also found after chronic treatment with ethanol and PB [21].

In conclusion, the data presented in our present and previous studies [10] indicate that the effects of DMI and possibly other tricyclic antidepressants on PB action depend on the interval between the administration of DMI and PB. Thus, acute treatment with DMI may potentiate the effect of PB shortly after administration of DMI to acute and chronic PB-treated rats. On the other hand, DMI may shorten the effect of PB after clearance of DMI from rats treated chronically with a combination of DMI and PB. Since tricyclic antidepressants and PB may be used concomitantly in man [11], the extrapolation of these findings to man would indicate that it is important to regulate the dose of PB according to the time of tricyclic antidepressant intake.

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7[^{14}C]pargyline binding to mitochondrial outer membranes*,†

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Studies *in vitro* using a highly purified bovine kidney enzyme have shown that 7[^{14}C]pargyline binds irreversibly and quite probably covalently to monoamine oxidase (MAO). The bound [^{14}C]pargyline was not extracted by a number of procedures including trichloroacetic acid (TCA) washes and chloroform: methanol extraction [1]. Under appropriate conditions, the radioactive inhibitor binds in a ratio of 1 mole/mole of MAO flavin, and the [^{14}C]pargyline can be recovered bound to a flavopeptide isolated from proteolytic digests of the inhibited enzyme [2]. It has also been reported that [^{14}C]pargyline is specific for MAO in the sense that it will not bind to other purified flavoproteins [1]. This argument has been extended by Erwin and Deitrich [3] who have shown that after its administration *in vivo* radioactive pargyline is distributed among the rat liver organelles roughly in parallel with their MAO content. These data and data *in vitro* suggest at least a degree of specificity in the binding of [^{14}C]pargyline to mitochondrial MAO.

In this report, the specificity of the binding has been further investigated using the interaction *in vitro* of [^{14}C]pargyline with mitochondrial outer membranes. The outer membrane preparations were isolated from the pooled livers of several 300 g male albino rats as described by Sottocasa *et al.* [4]. 7[^{14}C]pargyline‡ (7.03 μCi/mg) was the kind gift of Dr. A. O. Geiszler of Abbott Laboratories, North Chicago, Ill.

Usually about 0.4 to 0.8 mg/ml of outer membrane protein [5] was incubated at 35° in 50 mM Tris–HCl (pH 7.5) and varying concentrations of radioactive pargyline. In some experiments, the incubation was sampled and MAO activity was estimated at room temperature using a modification of the method of Tabor *et al.* [6]. In this assay, the benzylamine concentration was 1 mM and the buffer was 50 mM Tris–HCl (pH 7.5). One unit of enzyme activity is defined as 1 nmole benzaldehyde formed/min using a mM extinction coefficient for benzaldehyde of 13.8. The binding of [^{14}C]pargyline was estimated by measuring the

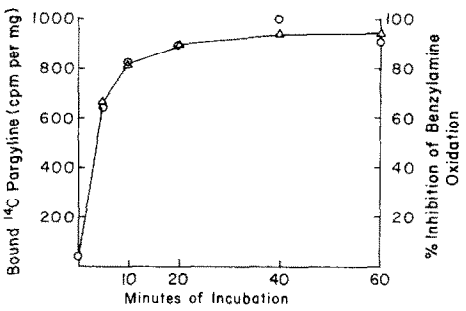

Fig. 1. Outer membranes (0.60 mg protein/ml) were incubated at 35° in 4 ml of a medium containing 50 mM Tris–HCl (pH 7.5) and 10^{-6} M 7[^{14}C]pargyline. At the indicated intervals, 0.1 ml was removed to measure the deamination of benzylamine (△—△) and 0.5 ml was removed to estimate bound radioactivity (○—○) by the TCA method (see text). Initially the specific enzymatic activity of MAO in the outer membranes was 93 units/mg of protein.

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‡ Radiochemical analysis by Abbott Laboratories showed that greater than 99.5 per cent of the radioactivity was present as pargyline.