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Disulfiram-like effect of diethyl maleate on barbiturate-induced hypnosis and 5-hydroxytryptamine metabolism

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Diethyl maleate (DEM, 600 mg kg^{-1} i.p.) significantly potentiated hexobarbitone hypnosis and lowered plasma hexobarbitone level on awakening. Sleeping time following intracerebroventricular (i.c.v.) injection of phenobarbitone was also prolonged by DEM treatment. When administered to DEM-treated rats, L-tryptophan (50 mg kg^{-1} i.p.) significantly potentiated hexobarbitone hypnosis, although alone it had no effect in control rats. DEM markedly diminished the activity of brain low-Km aldehyde dehydrogenase (AlDH) and the formation of 5-hydroxyindoleacetic acid from 5-hydroxytryptamine (5-HT) without affecting MAO activity in various areas of the brain. Conversely, the protein-bound radioactivity derived from i.c.v. [^{14}C]-5-HT was increased by DEM treatment. These results showed that DEM is comparable with disulfiram, a brain AlDH inhibitor, in terms of its effect on 5-HT metabolism and barbiturate hypnosis.

Diethyl maleate (DEM) is known to be an SH-conjugating agent (Boyland & Chasseaud 1967), but little or no information is available concerning its effect on the central nervous system. DEM is a potent inhibitor of brain low-Km aldehyde dehydrogenase (AlDH), which is an SH-enzyme and is responsible for the metabolism of biogenic aldehydes. Among these aldehydes, the metabolism of 5-hydroxyindoleacetaldehyde (5-HIAAld), derived from 5-hydroxytryptamine (5-HT), appears to depend largely on the activity of this enzyme, since 5-HT is metabolized mainly via the oxidative pathway to 5-hydroxyindoleacetic acid (5-HIAA), and to a minor extent by aldehyde reductase to 5-hydroxytryptophol (Duncan & Sourkes 1974).

On the other hand, it has been suggested that 5-HT metabolites could be involved in the sleep mechanism. Jouvet (1969) first postulated that deaminated metabolites of 5-HT may be implicated in the triggering of paradoxical sleep. Sabelli et al (1969) also demonstrated the sleep-inducing property of indoleacetaldehydes. Furthermore, the sedation and potentiation of hexobarbitone-induced hypnosis caused by prostaglandin E_1 (PGE_1) were shown to be related to the change in the 5-HT system or the increment of the brain level of 5-HT metabolites (Bhattacharya et al 1976; Haubrich et al 1973).

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Previously, we have demonstrated that disulfiram, another inhibitor of the brain AlDH, affects 5-HT metabolism, presumably through inhibition of the brain AlDH. The synergistic effects of disulfiram with a 5-HT precursor, or drugs that accelerate 5-HT synthesis, were demonstrated, suggesting the possible implication of 5-HIAAld in the modulation of barbiturate sensitivity by disulfiram (Fukumori et al 1980a; Minegishi et al 1981). The present study was designed to examine the effect of DEM on 5-HT metabolism and barbiturate-induced hypnosis.

MATERIALS AND METHODS

Male Wistar rats, 150–200 g, were housed under a 12 h light-dark schedule (8 h to 20 h) at 23 °C with free access to food and water.

Diethyl maleate was from Wako Pure Chemical Co., Tokyo, phenobarbitone sodium from Daiichi Chemical Co., Tokyo, hexobarbitone from Teikoku Chemical Co., Osaka, and propionaldehyde from Tokyo Kasei Co., Tokyo. β-NAD (grade II), L-tryptophan and 5-hydroxytryptamine (5-HT) oxalate were from Sigma, St Louis, MO, and [^{14}C]-5-HT and Aquasol from NEN, Boston, Mass.

Determination of the sensitivity to barbiturates

DEM, 600 mg kg^{-1}, was given 1 h before barbiturate injection. L-Tryptophan was given 15 min before hexobarbitone injection. Control rats were given the vehicles alone. Hexobarbitone (80 mg kg^{-1} i.p.) or phenobarbitone (1 mg/rat, i.c.v.) was injected

according to Minegishi et al (1979b). Barbiturate-induced sleeping time was determined by measuring the duration of loss of righting reflex. The plasma hexobarbitone concentration was determined as described by Minegishi et al (1979b).

Measurement of AlDH activity

Rats were decapitated and the whole brain rapidly removed. The enzyme source for AlDH assay was prepared by the method of Lebsack et al (1977) from the pellet after centrifugation at 10 000 *g*. The 40 000 *g* supernatant obtained in this procedure was further precipitated with 80% ammonium sulphate, and the resulting pellet was dissolved in sucrose solution (Lebsack et al 1977) and used for AlDH assay. AlDH activity was assayed as described by Minegishi et al (1979a). The reaction was initiated by addition of the substrate, propionaldehyde (3 mM or 50 μM).

Determination of MAO activity and in vitro 5-HIAA formation

Rats were decapitated 1 h after DEM or vehicle, and the brains immediately dissected into seven regions according to Glowinski & Iversen (1966). Tissue pieces were pooled and homogenized in 9 volumes of ice-cold 0·25M sucrose, and then centrifuged at 600 *g* for 10 min at 0 °C. The incubation mixture consisted of 0·25 mM [^{14}C]-5-HT (spec. act. 5 × 10^{-2}Ci mol^{-1}) and 1 mM NAD in 2 ml of 0·1 M phosphate buffer (pH 7·4). The mixture was incubated at 37 °C, and after 30 min the reaction was stopped by 1·5 ml of 3 M hydrochloric acid. Radioactive materials were extracted with 10 ml of ethyl ether. For determination of MAO activity, 3 ml of the ether extract was transferred to a counting vial and evaporated to dryness. After addition of 10 ml of Aquasol-2, the radioactivity was measured with a liquid scintillation counter. [^{14}C]-5-HIAA formed during the incubation was extracted from the residual ether extract. Five ml of the ether extract was added to a centrifuge tube containing 3 ml of 0·5M phosphate buffer (pH 7·0), shaken for 10 min, and then centrifuged at 3000 *g* for 5 min. After aspiration of the ether layer, the buffer layer was washed once with 3 ml of ether and the radioactivity measured.

Determination of the tissue binding of i.c.v. administered [^{14}C]-5-HT derived radioactivity

[^{14}C]-5-HT oxalate was administered i.c.v. at a dose of 500 nmol (0·75 μCi) per rat according to Fukumori et al (1979b). Thirty min later the rats were killed and the brains removed quickly and homogenized in 0·02 M EDTA solution. This homogenate was acidified with trichloroacetic acid (TCA, 10% final). After centrifugation, the precipitate was washed 5 times with 5% TCA. A part of the resultant pellet was used for the measurement of radioactivity in the TCA-insoluble fraction. The remaining portion of the pellet was extracted three times with chloroform –methanol, (2:1). This fractionation was conducted essentially according to Alivisatos & Ungar (1968). The resulting supernatant and pellet were used for determination of the radioactivity in the solvent-soluble and insoluble fractions, respectively.

RESULTS

DEM pretreatment significantly potentiated hexobarbitone hypnosis and lowered the brain hexobarbitone level on awakening (Table 1). These results indicate an increase in the brain sensitivity to barbiturates upon DEM treatment. This is further confirmed by the fact that the sleeping time following i.c.v. injection of phenobarbitone was significantly prolonged by DEM. As shown in Table 2, L-tryptophan alone did not affect the hexobarbitone sleeping time but it was significantly prolonged by L-tryptophan in DEM-pretreated animals.

Table 1. Effect of DEM treatment on barbiturate-induced hypnosis in rats.

Item	Control	DEM-treated	*P*
Hexobarbitone (80 mg kg^{-1}, i.p.)			
Sleeping time (min)	12·2 ± 1·7 (5)	20·3 ± 1·8 (5)	<0·01
Hexobarbitone concn on waking (μg ml^{-1} plasma)	47·6 ± 2·7 (4)	36·2 ± 2·6 (3)	<0·05
Phenobarbitone (1 mg/rat i.c.v.)			
Sleeping time (min)	15·2 ± 0·3 (5)	20·9 ± 1·0 (5)	<0·001

Figures in parentheses represent numbers of rats.

Previously, we reported that brain AlDH is highly susceptible to several inhibitors at low substrate concentration (Minegishi et al 1979a). Under the present experimental conditions (Table 3), AlDH was more markedly inhibited by DEM at the low substrate concentration either in vivo or in vitro. The inhibition of the enzyme at the high substrate concentration was significant only in vivo.

Table 2. Effect of tryptophan loading on hexobarbitone hypnosis in DEM-treated rats.

Pretreatment	L-Tryptophan	Sleeping time (min)	*P**
None	–	12·89 ± 0·89 (9)	—
	+	13·04 ± 0·82 (8)	n.s.
DEM	–	19·24 ± 1·10 (8)	—
	+	25·07 ± 0·75 (7)	<0·001

Figures in parentheses represent numbers of rats.
* *P*-values when compared with the corresponding values in the L-tryptophan (–) groups.

Table 3. Effect of DEM on rat brain AlDH activity in vivo and in vitro. In in vitro experiments, the reaction was initiated by addition of the substrate after preincubation with DEM (1 mM) for 5 min. Animals were killed 1 h after DEM treatment (600 mg kg^{-1} i.p.) in vivo. The concentrations of the substrate were 3·3 mM (high) and 50 μM (low). Each value is a mean ± s.e.m. of 4 or 5 determinations.

Substrate concn	Enzyme activity (nmol mg^{-1} protein/5 min)	
	Control	DEM
In vivo		
Low	3·84 ± 0·32	1·63 ± 0·27**
High	38·16 ± 0·97	30·12 ± 2·41*
In vitro		
Low	4·73 ± 0·13	1·87 ± 0·30**
High	44·33 ± 3·75	43·42 ± 3·16

* $P < 0·02$. ** $P < 0·001$.

To examine the metabolizing capacity for 5-HIAAld directly, in vitro 5-HIAA formation was observed in seven regions in the brain (Fig. 1). Although MAO activity with 5-HT as the substrate was highest in the hypothalamus, and lowest in the cortex, it was not changed by DEM treatment in any region in the brain. In contrast, in vitro 5-HIAA formation was markedly decreased in mid brain, medulla oblongata and cerebellum, and in the hypothalamus a slight but significant reduction of the 5-HIAA formation was also observed. In the other regions, the reduction of 5-HIAA formation was not significant. No correlation was apparent between MAO activity and change in 5-HIAA formation.

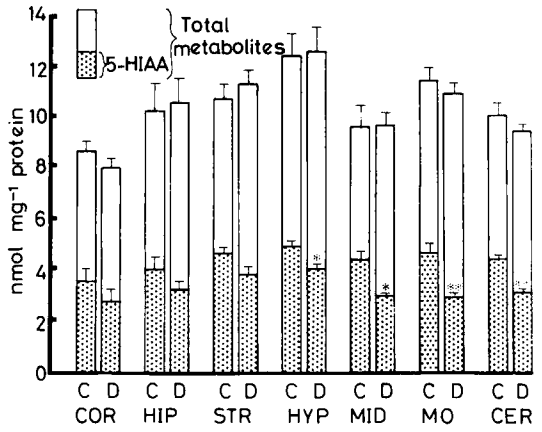

FIG. 1 Effect of DEM treatment on MAO activity and in vitro 5-HIAA formation in seven regions of the rat brain. Rats were treated with DEM (600 mg kg^{-1}, i.p.) or the vehicle. After 30 min, they were killed and MAO activity and 5-HIAA formation were determined as described in 'materials and methods'. The seven regions of the brain are as follows: COR indicates cortex; HIP, hippocampus; STR, striatum; HYP, hypothalamus; MID, midbrain; MO, medulla oblongata; CER, cerebellum. Treatments are: C, control; D, DEM treatment.
* $P < 0·02$. ** $P < 0·01$.

Biogenic aldehydes are known to bind to brain tissue. The measurement of the binding level may help, indirectly, in estimating the 'steady state level' (Deitrich & Erwin 1975) in the brain. Some radioactivity derived from i.c.v. administered [^{14}C]-5-HT remained in the TCA-insoluble material (Table 4), which corresponds to protein-bound activity. The TCA-insoluble fraction was further divided into chloroform–methanol-soluble and -insoluble fractions. DEM pretreatment increased the radioactivity in each fraction. The greatest increase (34·3%) was observed in the chloroform–methanol-soluble fraction, which corresponds to proteo-lipid material.

Table 4. Effect of DEM on the incorporation of radioactivity derived from i.c.v. injected [^{14}C]-5-HT into TCA and chloroform–methanol (CM)-treated fractions of rat brain.

Treatment	CM-soluble (nmol g^{-1} brain)	Radioactivity (1 × 10^{-10} mol mg^{-1} protein)	
		TCA-insoluble	CM-insoluble
None	5·28 ± 0·45	1·46 ± 0·07	1·17 ± 0·09
DEM	7·09 ± 0·62*	1·70 ± 0·13**	1·28 ± 0·14
% increase	34·3	16·4	9·4

Rats were pretreated with DEM (600 mg kg^{-1} i.p.) 1 h before [^{14}C]-5-HT injection (500 nmol, 0·75 μCi) i.c.v. Animals were killed 30 min after 5-HT injection. Values represent means ± s.e.m. from 8 trials.
Significantly different from control *($P < 0·05$), **($P < 0·01$).

DISCUSSION

DEM potently decreased the brain low-Km AlDH activity (Table 3). The disturbance of 5-HIAAld metabolism is evident from Fig. 1. MAO activity was not affected by DEM treatment in any region examined. However, 5-HIAA formation was significantly diminished in four regions. This result indicates that the inhibition of the enzyme resulted in the reduction of 5-HIAA formation from 5-HIAAld without affecting the 5-HIAAld formation.

5-HIAAld cannot be detected directly, since it is a transient metabolite that binds easily to brain tissue. It has been shown that firmly bound materials derived from biogenic amines in vitro are aldehydes derived from their parent amines (Alivisatos & Ungar 1968). Also, when labelled 5-HT was injected i.c.v., the bulk of the incorporation in vivo into acid-insoluble material of the brain is due to the aldehyde, 5-HIAAld (Alivisatos et al 1970). Thus, under our experimental conditions, where the MAO activity was not altered by DEM, increase in the radioactivity incorporated into TCA-insoluble

material, i.e. protein-bound radioactivity, may actually reflect the increment of brain 5-HIAAld level. Furthermore, the results in Table 4 show that the increase in the incorporation into this fraction is largely due to the increase into chloroform–methanol-soluble material, which contains proteolipid material, such as synaptosomal membrane (Ungar & Alivisatos 1976). Alivisatos & Ungar (1968) suggested the incorporation of 5-HIAAld was important for modifying the membrane properties and in the neuromodulatory effect of 5-HIAAld (Ungar & Alivisatos 1976). Our results suggest that an increased amount of 5-HIAAld binds to the synaptosomal membrane in DEM-treated rats, as a result of the reduction of the synaptosomal low-Km AlDH activity, and possibly affects the neuronal activity of the 5-HT system and behavioural expression.

There is a considerable agreement between the observed effect of DEM on 5-HIAAld metabolism and that of disulfiram. We have shown that disulfiram potently inhibits the brain low-Km AlDH (Minegishi et al 1979a), reduces the formation of 5-HIAA in vitro (Fukumori et al 1980b) and in vivo (Fukumori et al 1979a), and increases the binding of the 5-HT metabolite to brain tissue (Fukumori et al 1978). Thus, the similarity between the effects of these drugs seems to be attributable to their inhibitory effect on AlDH.

DEM markedly prolonged the barbiturate-induced sleeping time (Table 1). The enhancement of the brain sensitivity to barbiturate by DEM was confirmed by the reduction of the hexobarbitone concentration on awakening and prolongation of the phenobarbitone (i.c.v.) sleeping time. The potentiating effect of DEM on hexobarbitone hypnosis was augmented by tryptophan, which on its own had no effect on hexobarbitone hypnosis (Table 2). This indicates that modification of the 5-HIAAld metabolism is presumably involved in the prolongation effect of DEM on barbiturate-induced sleeping time.

Recent work showed that disulfiram significantly potentiates barbiturate-induced hypnosis (Minegishi et al 1979b) and that the prolongation of hexobarbitone hypnosis by disulfiram is enhanced by L-tryptophan (Minegishi et al 1981). In addition, disulfiram, in combination with drugs that increase 5-HT synthesis, i.e. lithium and PGE_1, synergistically prolongs barbiturate hypnosis (Fukumori et al 1980a; Minegishi et al 1981). On the basis of these findings, we previously suggested that the increase in 5-HIAAld by disulfiram, resulting from inhibition of brain AlDH, may be causally related to the modulation of barbiturate sensitivity by disulfiram. We are tempted to speculate that the increase in 5-HIAAld level produced by DEM may be partly responsible for the prolongation of barbiturate hypnosis by DEM, and that the combination of DEM with L-tryptophan, by increasing the formation of 5-HIAAld and reducing its metabolism, caused the synergistic enhancement of barbiturate-induced sleeping time.

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