Biochemical Pharmacology, 1965, Vol. 14, pp. 305–311. Pergamon Press Ltd., Printed in Great Britain.

PROLONGATION OF HEXOBARBITAL SLEEPING TIME AND INHIBITION OF HEPATIC DRUG METABOLISM BY ADRENERGIC BLOCKING AGENTS IN MICE*

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(*Received* 17 *August* 1964; *accepted* 16 *October* 1964)

Abstract—The adrenergic blocking agents tolazoline, hydralazine, phentolamine, phenoxybenzamine, azapetine, and yohimbine prolonged hexobarbital sleeping time in mice at room temperature. All these drugs demonstrated a rapid onset and short duration of action with regard to their effect on sleeping time. Keeping mice in an oven set at 30–32° reversed the hypothermic effects of these drugs and partially or completely reversed the prolongation of sleeping time which was seen at room temperature. Significant prolongation of sleeping time at elevated environmental temperature was produced only by pretreatment with hydralazine, phentolamine, phenoxybenzamine, and azapetine. Only the last four of the six drugs studied also inhibited various oxidative pathways of hepatic drug metabolism *in vitro*.

THE PRESENT work is a continuation of studies in this laboratory on the inhibition of hepatic microsomal drug metabolism by norepinephrine;[1] its purpose is to demonstrate prolongation of hexobarbital sleeping time in mice by various adrenergic blocking agents, to point out that hypothermia as well as direct inhibition of drug metabolic enzymes may lead to prolongation of hexobarbital sleeping time, and to correlate these *in-vivo* findings with results from studies on drug metabolism *in vitro*.

MATERIALS AND METHODS

Drugs

Tolazoline (Priscoline, CIBA†), hydralazine hydrochloride (Apresoline, CIBA†), phenoxybenzamine (Dibenzyline, Smith, Kline, & French†), and yohimbine hydrochloride (K&K) were the adrenergic blocking agents used. Sodium hexobarbital (Evipal-soluble, Winthrop) was used to induce sleep.

Animals

Male Sutter mice weighing 25–30 g were used. The mice were on hand 4 to 14 days prior to use and were maintained on Wayne lab block.

In vivo determinations

The "hexobarbital sleeping time" is the duration of the loss of the righting reflex after i.p. injection of hexobarbital at a dose of 80–100 mg/kg in a volume of 10 ml/kg body weight.

* This research was supported by Grant CA-05648 from the National Institutes of Health.
† Our thanks to the above companies who so generously provided these drugs free of charge.

Body temperatures were determined with a Yellow Springs telethermometer and rectal probes. The mice were taped to a tongue depressor, and the rectal probes were maintained in place by taping them to the tails of the mice. Because the activity of most enzymes is temperature dependent, a drop in body temperature could prolong hexobarbital sleeping time by decreasing the rate of metabolism of the barbiturate.

Hexobarbital sleeping times were also determined in mice kept in an oven set at 30–32°. At this temperature, control and treated mice were maintained at normal body temperatures (37–38°).

In vitro determinations

The mice were sacrificed by a blow on the head. As soon as possible, their livers were removed and placed on ice. The gall bladders were excised and the livers were weighed, 2 ml of cold 1·15% KCl was added per g tissue, and the mixture was homogenized in the cold with a Potter homogenizer having a plastic pestle. A supernatant fraction, containing microsomal and soluble enzymes, was prepared from the homogenate by centrifugation at 9,000 *g* for 20 min at 5°.

One ml of 9,000 *g* supernatant fraction was incubated with cofactors and substrate for 90 min in a Dubnoff shaking incubator at 37° with oxygen as the gaseous phase. Final concentrations of cofactors added were: triphosphopyridine nucleotide (NADP) ($1{\cdot}1 \times 10^{-4}$ M), glucose-6-phosphate (5×10^{-3} M), nicotinamide (2×10^{-2} M), and $MgSO_4$(5×10^{-3} M). All concentrations are supraoptimal. The final volume of all incubation mixtures was brought up to 5 ml with 0·1 M phosphate buffer, pH 7·4.

The pathways studied and substrate concentrations in micromoles per 5-ml incubate were: side-chain oxidation of hexobarbital (3 μmoles) to ketohexobarbital, the N-dealkylation of aminopyrine (40 μmoles) to 4-aminoantipyrine, the o-dealkylation of codeine (10·2 μmoles) to morphine, and the hydroxylation of aniline (5·0μmoles) to *p*-aminophenol. Disappearance of hexobarbital was measured by the method of Cooper and Brodie.[2] The appearance of 4-aminoantipyrine, morphine, and *p*-aminophenol was measured by methods of Brodie and Axelrod,[3] Snell and Snell, [4] and J. R. Gillette (personal communication) respectively. For the *p*-aminophenol determination, 2 g of NaCl was added to a 4-ml aliquot of the incubate, and the mixture extracted by shaking with 30 ml ether for 20 min. Twenty ml of the ether phase was then extracted with 4 ml 1 N Na_2CO_3 containing 1% phenol (freshly prepared). One ml of a freshly prepared bromine–Na_2CO_3 mixture (bromine water added to 1 N Na_2CO_3 until a light yellow color appears) was added to 3 ml of the Na_2CO_3 phase, and the mixture read at 620 mμ.

Statistical evaluation of data

The statistical methods used are described by Snedecor.[5] The Student's "t" distribution was used as a test of the null hypothesis. The level of significance used in all determinations was P equal to or less than 0·05. All values expressed in tables are means plus or minus standard deviation.

RESULTS

In vivo

All the adrenergic blocking agents tested significantly prolonged hexobarbital

sleeping time of animals kept at room temperature (25–26°) after a single injection given 30 min prior to the hexobarbital (Table 1). The dose–response relationship of each drug was studied over a wide range of doses with the maximum effect being a prolongation of sleeping times to 150–250% that of control values.

TABLE 1. PROLONGATION OF HEXOBARBITAL SLEEPING TIME BY VARIOUS DOSES OF ADRENERGIC BLOCKING AGENTS

Dose† (mg/kg)	Sleeping time*					
	Tolazoline	Hydralazine	Phentolamine	Phenoxybenzamine	Azapetine	Yohimbine
100	*50 ± 16*	*58 ± 19*				
50	*41 ± 13*	*34 ± 9*	*50 ± 19*			
40				*110 ± 16*		
25	*39 ± 14*	*34 ± 8*				
20			*40 ± 24*	*86 ± 22*		*131 ± 17*
10	*30 ± 9*	23 ± 7	20 ± 7	*71 ± 24*	*55 ± 17*	*56 ± 27*
5	*38 ± 10*	26 ± 4	34 ± 17	*87 ± 40*	*48 ± 17*	*39 ± 10*
2·5	*43 ± 7*	26 ± 8	30 ± 5	*81 ± 13*	*34 ± 8*	34 ± 10
1	*38 ± 10*	22 ± 8	27 ± 18	*60 ± 14*	24 ± 9	36 ± 15
0·5		26 ± 8	27 ± 8	*45 ± 11*	*32 ± 9*	39 ± 19
Control	18 ± 7	19 ± 5	24 ± 8	37 ± 8	22 ± 8	28 ± 7

* Duration (min) of the loss of the righting reflex after i.p. injection of hexobarbital at a dose of 80 mg/kg, except for the phenoxybenzamine group which received a dose of 100 mg/kg. Each value represents the mean ± standard deviation of a group of 7–10 mice. Significant results are italicized ($P<0.05$).

† Drug injected in aqueous solution i.p. 30 min prior to hexobarbital injection, in a volume of 10 ml/kg.

TABLE 2. PROLONGATION OF HEXOBARBITAL SLEEPING TIME BY ADRENERGIC BLOCKING AGENTS: DURATION OF ACTION

Time of drug injection with respect to time of administration of hexobarbital†	Sleeping time*					
	Tolazoline, 50 mg/kg	Hydralazine, 50 mg/kg	Phentolamine, 50 mg/kg	Phenoxybenzamine, 20 mg/kg	Azapetine, 5 mg/kg	Yohimbine, 10 mg/kg
10 min after	22 ± 10	15 ± 3	24 ± 16	*48 ± 12*	18 ± 8	19 ± 6
0 min after	*30 ± 13*		*27 ± 6*	*50 ± 10*	*39 ± 19*	15 ± 5
15 min before	*30 ± 7*	*27 ± 8*	*27 ± 9*	*54 ± 10*		*52 ± 26*
30 min before	*31 ± 13*	*34 ± 15*	*30 ± 12*	*69 ± 11*	*51 ± 17*	*40 ± 18*
60 min before	*30 ± 8*	*53 ± 13*	*52 ± 21*	*118 ± 11*	*39 ± 11*	*34 ± 12*
90 min before	*39 ± 15*	*47 ± 3*	26 ± 14	*91 ± 33*	20 ± 7	22 ± 6
3 hr before	24 ± 6	20 ± 8	18 ± 5	*82 ± 19*	13 ± 4	21 ± 10
6 hr before	27 ± 9	26 ± 17	15 ± 6	*89 ± 24*		16 ± 2
12 hr before	24 ± 10	26 ± 16	20 ± 8	*75 ± 20*	18 ± 2	21 ± 6
24 hr before		21 ± 10		*69 ± 16*	13 ± 3	19 ± 7
48 hr before	20 ± 9	23 ± 5	18 ± 5	31 ± 5	17 ± 6	23 ± 9
Control	19 ± 6	18 ± 7	14 ± 4	33 ± 9	15 ± 4	20 ± 5

* Duration (min) of the loss of the righting reflex after i.p. injection of hexobarbital at a dose of 80 mg/kg, except for the phenoxybenzamine group which received a dose of 100 mg/kg. Each value represents the mean ± standard deviation of a group of 7–10 mice. Significant results are italicized ($P<0.05$).

† Drug injected i.p. in aqueous solution at the given time and dose.

Each of the drugs studied exhibited a rapid onset and short duration of action in prolonging hexobarbital sleeping times (Table 2). None of the drugs, except phenoxybenzamine, consistently prolonged sleeping times if injected more than 90 min prior to hexobarbital anesthesia.

None of the adrenergic blocking drugs studied induced loss of righting reflex when injected alone or when administered to animals which had just regained the righting reflex after hexobarbital anesthesia. None would therefore seem to have sensitized the central nervous system to hexobarbital.

All the adrenergic blocking agents lowered body temperature 2–4° below control body temperatures over a 90-min period. Increasing the temperature of the environment by placing the mice in an oven at 30–32° seemed to block this temperature effect and maintained the body temperature of treated animals at control levels. Figure 1 is typical of the results we obtained for the drugs studied. By such experiments we

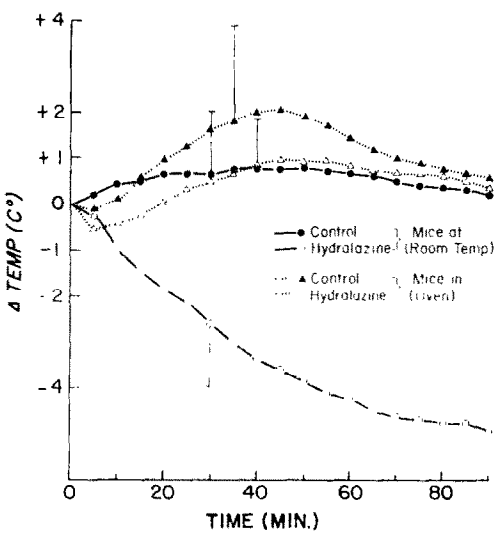

FIG. 1. Hypothermic effect of hydralazine in mice and the inhibition of this effect by maintaining mice in an oven set at 30–32°. Hydralazine was injected i.p. at a dose of 50 mg/kg.

hoped to distinguish between adrenergic blocking agents affecting sleeping times by inhibiting microsomal enzymes and those acting by lowering body temperature.

Hexobarbital sleeping times measured in control and treated mice maintained at normal body temperatures in the oven were compared with sleeping times of mice kept at room temperature (Table 3). Mice treated with various adrenergic blocking agents and kept at room temperature after hexobarbital injection all slept for times which differed significantly from controls given hexobarbital alone (Tables 1, 2, and 3). Phentolamine, hydralazine, phenoxybenzamine, and azapetine were still able to prolong hexobarbital sleeping times in mice kept at 30–32°, but such was not true with tolazoline or yohimbine (Table 3). Control mice (hexobarbital only) kept at room temperature did not differ significantly (in sleeping time) from controls kept in the oven (30–32°).

TABLE 3. PROLONGATION OF HEXOBARBITAL SLEEPING TIME IN MICE BY ADRENERGIC BLOCKING AGENTS WHEN CONTROL AND TREATED MICE ARE MAINTAINED AT NORMAL BODY TEMPERATURES AS COMPARED TO MICE KEPT IN A ROOM TEMPERATURE ENVIRONMENT

Experimental groups	Dosage of drug* (mg/kg)	Environment†	Sleeping time‡	N
1. Control		Room temp.	23 ± 3	4
2. Tolazoline	50	Room temp.	46 ± 21§	4
3. Control		Oven	18 ± 4	4
4. Tolazoline	50	Oven	22 ± 3	4
5. Control		Room temp.	19 ± 5	5
6. Yohimbine	10	Room temp.	38 ± 17§	5
7. Control		Oven	18 ± 8	5
8. Yohimbine	10	Oven	20 ± 7	5
9. Control		Room temp.	17 ± 7	5
10. Hydralazine	50	Room temp.	47 ± 3§	5
11. Control		Oven	18 ± 6	5
12. Hydralazine	50	Oven	29 ± 8§	5
13. Control		Room temp.	21 ± 12	4
14. Azapetine	10	Room temp.	55 ± 10§	4
15. Control		Oven	23 ± 2	4
16. Azapetine	10	Oven	32 ± 4§	4
17. Control		Room temp.	22 ± 10	4
18. Phenoxybenzamine	20	Room temp.	69 ± 16§	4
19. Control		Oven	22 ± 11	4
20. Phenoxybenzamine	20	Oven	49 ± 4§	4
21. Control		Room temp.	18 ± 6	5
22. Phentolamine	50	Room temp.	31 ± 10	5
23. Control		Oven	18 ± 7	5
24. Phentolamine	50	Oven	29 ± 8§	5

* Drug injected i.p. in aqueous solution 30 min prior to hexobarbital injection.
† Room temperature was 24–25°; oven temperature was 30–32°.
‡ Duration (min) of the loss of the righting reflex after i.p. injection of hexobarbital, 80 mg/kg. Each value represents the mean ± standard deviation.
§ $P < 0.05$ when treated animals compared with controls at the same environmental temperature.

In vitro

Various molar concentrations (from 10^{-5} to 10^{-3}) of the adrenergic blocking agents were added to incubation media to determine the concentration of drug that would reduce the metabolism of a given substrate to 50% of the control value (I_{50}; Table 4). Of the drugs studied, only phenoxybenzamine, hydralazine, and azapetine significantly inhibited drug metabolism *in vitro* at concentrations less than 10^{-3} M. Phentolamine also exhibited inhibitory effects, but I_{50} values were greater than 10^{-3} M.

The metabolic pathways studied were not inhibited to the same degree by a given adrenergic blocking agent, but the variation of I_{50} values for the different routes of drug metabolism was not large (Table 4).

Inhibition of microsomal drug metabolism by phenoxybenzamine and hydralazine added *in vitro* is similar in magnitude to the inhibition by chloramphenicol and

TABLE 4. MOLAR CONCENTRATION OF ADRENERGIC BLOCKING AGENTS PRODUCING 50% INHIBITION OF VARIOUS PATHWAYS OF HEPATIC MICROSOMAL DRUG METABOLISM IN MICE*

Substrate	Product	Inhibitor concentration (M × 10^{-4})		
		Phenoxybenzamine	Hydralazine	Azapetine
Hexobarbital	Ketohexobarbital	1·0 ± 0·2	6·6 ± 7·3	0·88 ± 0·25
Aminopyrine	4-Aminoantipyrine	3·4 ± 1·3	4·4 ± 2·6	2·3 ± 0·3
Aniline	*p*-Aminophenol	9·4 ± 4·0	10·9 ± 4·7	No inhibition†
Codeine	Morphine			4·8 ± 0·8

* Each value represents the molar concentration of drug ± standard deviation in the final incubation mixture. Each mean is the result of three determinations.

† No inhibition seen at highest concentration used (1 × 10^{-3} M).

SKF 525-A reported earlier.[6] Azapetine is interesting because it does not seem to inhibit the *p*-hydroxylation of aniline, although it does inhibit the other pathways studied.

DISCUSSION

All the adrenergic blocking agents studied prolonged hexobarbital sleeping time in mice, but only hydralazine, phenoxybenzamine, phentolamine, and azapetine could be shown to directly inhibit microsomal drug metabolism *in vitro* (Tables 1 through 4). Prolongation of hexobarbital sleeping times could be the result of a decreased rate of metabolism of the barbiturate. Such a decreased rate of metabolism of hexobarbital could be caused by both a direct inhibition of the microsomal enzymes by the adrenergic blocker and a depression of body temperature. We feel that this latter mechanism is shared by all the adrenergic blocking agents studied, whereas the direct inhibition of microsomal enzymes could be shown only with hydralazine, phenoxybenzamine, azapetine, and phentolamine, Thus, raising environmental temperature to a point that maintained body temperature at normal levels (37–38°) even in the presence of drugs left only hydralazine, phenoxybenzamine, azapetine, and phentolamine still able to prolong sleeping time when compared with controls at the same temperature. The prolongation of hexobarbital sleeping time by yohimbine and tolazoline (seen at room temperature) is not seen at an environmental temperature of 30–32°.

The results of our experiments *in vitro* were consistent with our results *in vivo*. Hydralazine, phentolamine, phenoxybenzamine, and azapetine all inhibited oxidative drug metabolism *in vitro*, but phentolamine was a relatively weak inhibitor.

Using kinetic analysis (Lineweaver-Burk plots), we attempted to determine the nature of the inhibition of drug metabolism by adrenergic blocking agents, but the results were variable and we were unable to interpret them. Both competitive and noncompetitive inhibition (as defined by Michaelis-Menten equations) were observed with all inhibitors. The nature of the inhibition may depend on the substrate used and we hope to investigate this further.

Acknowledgements—The authors wish to thank the following for technical assistance: Mr. Richard Hughes, Mr. Arnold Balanoff, Mr. Jerry Blakely, Mrs. Bobbi Pohl, Mrs. Diana Lewiston, and Mrs. Barbara Purdie.

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