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EFFECT OF VARYING PERIODS OF COLD EXPOSURE ON THE ACTION AND METABOLISM OF HEXOBARBITAL*

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Abstract—This study compares the hypnotic effect of hexobarbital with its rate of metabolism *in vitro* in the rat exposed to cold (5°) for varying periods of time. Whereas the 25° control rats showed a decrease in sleep time that was most pronounced after 1–3 weeks of exposure and which corresponded with an increased rate of metabolism, the cold-exposed rats showed longer sleep times than their age-matched controls as early as 3 days after exposure which persisted for the 7 weeks of the experiment. The rate *in vitro* of hexobarbital metabolism of the cold-exposed groups did not vary significantly from the zero day control level during the entire experiment. Brain levels of hexobarbital upon awakening were the same for control rats as for rats exposed to 5° for 3 days. A seasonal effect to the hypnosis is suggested, since rats subjected to 3 days of cold exposure in April exhibited a changed dose–response curve from that of their warm controls, but this did not occur for similar groups tested in November.

EXPOSURE to low environmental temperatures may alter drug metabolism.[1–4] Changes in drug response[5, 6] and in drug toxicity[7] occur upon exposure to cold environments. A correlation between these changes in drug response and drug metabolism might therefore be anticipated for physical agents such as cold, since such a correlation has already been shown for a wide variety of chemical agents.[8] In a preliminary report, Fuller and Bousquet[4] describe such a finding. There is reason to believe that the regulatory processes elicited during cold exposure vary during the time of that exposure; e.g. shivering constitutes the main mode of increased heat production in rats during the initial exposure to cold but not when that exposure is continued for a period of 2 weeks or longer. At this period, nonshivering thermogenesis[9] becomes the most important mode of heat production. In what follows, we report the results of experiments that were designed to follow the changes in hexobarbital potency and the metabolism of the drug *in vitro* during different durations of cold exposure.

EXPERIMENTAL

Male, Wistar rats, obtained at 30 ± 3 days of age, were placed in individual wire-mesh cages in rooms of either $5° \pm 2°$ or $25° \pm 1°$ for periods from 1 to 49 days. All rats were maintained on Rockland Mouse and Rat Diet and water *ad libitum* up to and including the experiment day. Rats were weighed 3 times weekly during the entire exposure period. The mortality for the rats exposed to 5° was

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approximately 11 per cent for the 7-week period; rats exposed to 25° showed a mortality of less than 1 per cent. Rats were injected i.p. with hexobarbital sodium immediately after removal from their respective environments and sleeping times were measured at room temperature of about 23°. A different set of rats was run on each test day. The injected volume of drug was always 5 ml/kg and, except where otherwise noted, the injected dose was always 100 mg/kg of the sodium salt of hexobarbital, freshly prepared for each experiment. Sleeping time was determined as that period during which the rat was unable to right itself twice in succession after being placed on its back. Rats in which the onset of action was longer than 5 min were discarded as possibly due to a faulty injection.

Rate of hexobarbital metabolism by the 9000 *g* liver supernatant was estimated from a 30-min incubation period at 37° under 95% oxygen–5% CO_2. In experiment 1 of Table 1, pooled liver samples were used; in experiment 2 of the same table, individual samples were used and the experiment was subdivided into groups of the following time periods: 0–7 days, 2–4 weeks and 5–7 weeks. Each estimation was done in duplicate. Rats were killed by stunning followed by cervical dislocation. Livers were removed, blotted, chilled and immediately homogenized in cold, isotonic KCl (1·15%, 2·0 ml/g liver) in a Potter homogenizer with a plastic pestle. Drug metabolism was assayed on the supernatant obtained after a 20-min centrifugation at 9000 *g* at 5°. The incubation mixture was composed of the following solutions, used in the volumes indicated and in successive order: 0·1 M, pH 7·4 phosphate buffer (2·3 ml); 0·1 M nicotinamide–0·05 M $MgSO_4$–0·02 M D-glucose 6-phosphate (1·0 ml); 0·006 M hexobarbital sodium (0·5 ml); 0.0025 M NADP (0·2 ml) and 9000 *g* supernatant (1·0 ml). The amount of unchanged hexobarbital remaining at the termination of the incubation period was assayed by the method of Cooper and Brodie.[10]

Brain levels of hexobarbital were assayed in some experiments, either at the time of awakening or at the time of death. After decapitation, the entire brain was removed, frozen on dry ice and assayed the following day. The weighed tissue was homogenized with 0·001 M NaOH (3 ml/g brain) and 4 ml of the homogenate was immediately added to 0·5 M, pH 5·5 citrate buffer (4·0 ml), which was sufficient to buffer the added alkali. Hexobarbital was then extracted into heptane–isoamyl alcohol as for the liver extraction.

Preliminary control experiments on the effect of a pyrethrin-containing insecticide (Milfuso) ruled out the possibility that effects of the cold exposure were due to an absence of this insecticide. The warm room had been occasionally sprayed during April 1966, but not thereafter, with this insecticide. The pyrethrins have never been implicated as enzyme-inducing insecticides,[11] but we felt it necessary to confirm this for the insecticide that was used. Accordingly, the insecticide was used in a much more intensive manner than was its actual use; it was sprayed directly on the animals, cages and food daily for the 3 days during which all animals remained in the 25° room and for 3 additional days only on those animals remaining at 25°. A second comparable group was protected from all insecticide but exposed to the same environmental conditioning. There was an approximate doubling of the sleeping time of the cold-exposed groups compared with their respective warm groups under both regimens (these data appear in Table 3).

Plasma and liver proteins were determined, after appropriate dilutions, by the microbiuret method of Itzhaki and Gill.[12] Blood was obtained by cardiac puncture just

prior to removal of the liver. After microhematocrits were obtained, the plasma was frozen until analysis, usually 24 hr later. Aliquots of the whole liver homogenate and of the 9000 *g* supernatant were also frozen and analyzed within a few days.

Statistical comparisons were made by Student's *t*-test as described by Snedecor.[13]

RESULTS

Growth curves. The slower rate of growth of rats housed at 5° compared with rats housed for comparable periods at 25° is apparent from Fig. 1 (upper half). Liver weight, expressed as a percentage of body weight, is greater for the cold than for the warm-exposed rat (Fig. 1, lower half). However, both curves have a similar contour, increasing up to a maximum at about 2 weeks (rats 44 days old) and decreasing at the later ages. At an equivalent age, the absolute liver weight is lower for the cold than for the warm-exposed rats (9·25 g vs. 14·43 g at 5 weeks of exposure). Blood hematocrits increased as the rat matured (33 ± 1·6 up to 46 ± 2·7), but differences between warm and cold groups of comparable age were insignificant. Means for plasma proteins varied between 58·5 ± 5·22 (S.E.) and 75·5 ± 2·95 mg/ml; these values did not show age-related or exposure-related differences.

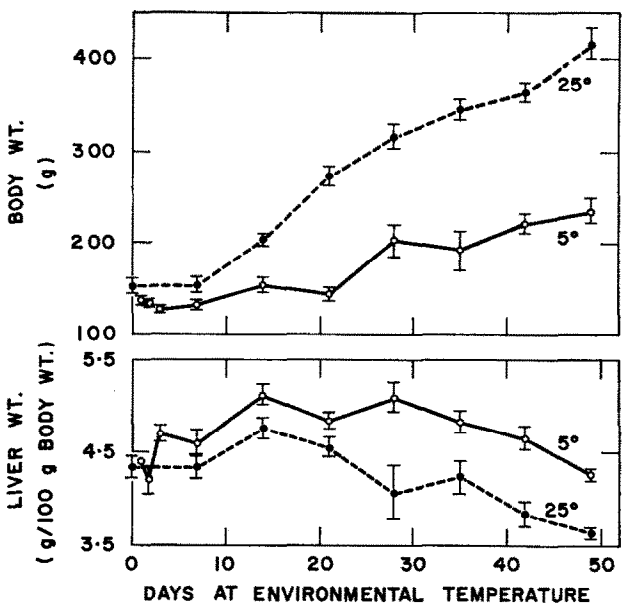

FIG. 1. Effect of the environmental temperature on body weight and liver weight. Each point represents the mean ± S.E. from 4–5 male, Wistar rats. Age of rats on day 0 of experiment is 30 ± 3 days.

Hypnotic action of hexobarbital. Rats exposed to 5° for at least 3 days and for as long as 7 weeks (the duration of the experiment) sleep longer than their age-matched 25° controls (Table 1). The 25° control groups show a decrease in sleeping time which is especially marked between 1 and 3 weeks and which persists for the entire experiment. The longer sleep times evident after 3 days of cold exposure are also apparent in the data of Table 2 (April 24, 1967) and of Tables 3 and 4.

The dose–response relationship for hexobarbital sleeping time after 3 days of cold

TABLE 1. EFFECT OF VARYING PERIODS OF COLD EXPOSURE ON SLEEPING TIME AND ON HEPATIC METABOLISM OF HEXOBARBITAL*

Exposure†	Trial‡	Sleep time§ (min) ± S.E.		Hexobarbital metabolized‖ (μg/g/30 min)	
		Control	Cold-exposed	Control	Cold-exposed
1 day	1	29 ± 3·5 (5)	30 ± 2·7 (5)	0·64 ± —	0·56 ± —
	2	22 ± 0·8 (5)	23 ± 2·7 (5)	0·61 ± 0·37	0·60 ± 0·078
2 day	1		37 ± 3·5 (5)		0·59 ± —
	2		24 ± 0·6 (4)		0·82 ± 0·096
3 day	1		38 ± 5·1 (5)		0·49 ± —
	2		43 ± 6·0 (5)¶		0·74 ± 0·033
1 week	1	22 ± 2·1 (5)	39 ± 1·2 (5)**	0·54 ± —	0·48 ± —
	2	24 ± 4·7 (5)	35 ± 2·5 (5)	0·84 ± 0·079	0·72 ± 0·060
2 week	1	18 ± 4·0 (5)	27 ± 3·6 (5)	0·84 ± —	0·80 ± —
	2	12 ± 1·2 (5)	37 ± 3·4 (5)**	0·90 ± 0·050	0·62 ± 0·076¶
3 week	1	12 ± 0·8 (5)	24 ± 2·5 (5)**	0·97 ± —	0·70 ± —
	2	13 ± 2·4 (5)	25 ± 1·5 (5)**	0·92 ± 0·058	0·78 ± 0·058
week	1	12 ± 0·8 (5)	21 ± 2·1 (5)**	1·05 ± —	0·71 ± —
	2	12 ± 1·1 (5)	33 ± 4·9 (5)**	0·98 ± 0·076	0·56 ± 0·040**
5 week	1	15 ± 1·4 (5)	32 ± 3·4 (5)**	0·90 ± —	0·59 ± —
	2	15 ± 1·5 (5)	48 ± 1·6 (5)**	1·24 ± 0·050	0·70 ± 0·024**
6 week	1	18 ± 2·3 (3)	25 ± 5·1 (3)	0·98 ± —	0·62 ± —
	2	16 ± 1·0 (5)	43 ± 2·9 (5)**	1·25 ± 0·060	0·94 ± 0·093¶
7 week	1	17 ± 0·6 (4)	24 ± 3·5 (5)	0·92 ± —	0·83 ± —
	2	17 ± 1·6 (5)	35 ± 3·4 (4)**	1·02 ± 0·087	0·78 ± 0·058

* Fed, male Wistar rats injected i.p. with 100 mg/kg of hexobarbital sodium in a volume of 5 ml/kg.

† Rats raised at 25° exposed at 30 ± 3 days of age to environmental temperatures of 25° (control) or 5° (cold-exposed) for lengths of time designated.

‡ Trial 1 (4/1/66) = livers pooled for analysis of hexobarbital metabolism; trial 2 (4/27/66) = livers analyzed individually.

§ Time between loss of and regaining of righting reflex. Number of rats is in parentheses.

‖ Hexobarbital metabolized by the 9000 *g* supernatant of liver, as measured by disappearance of substrate from incubation medium described in Methods. Each sample was analyzed in duplicate.

¶ Cold-exposed group significantly different from corresponding control of same age at $P < 0.05$.

** Cold-exposed group significantly different from corresponding control of same age at $P < 0.01$.

exposure (Table 2, data of April 1967) shows that sleeping times are approximately doubled at the 3 dose levels examined. There is also the suggestion of an increased mortality in the cold-exposed rats. The dose–response curve of November 1966, will be discussed in a later section.

Metabolism of hexobarbital. While the control groups show a marked increase in their relative rates of metabolism of hexobarbital (*in vitro*) between the first and third weeks, the cold-exposed groups maintain a relatively constant rate of metabolism throughout, and one which is significantly lower than that of their age-matched controls during the 4–6 weeks (Table 1). Although statistical comparisons can only be obtained for experiment 2, in which individual livers rather than pooled livers were assayed, the general pattern of response appears to be similar for the two trials. Hexobarbital metabolism is expressed on a liver wet weight basis. This is possible because there was no marked difference in protein content of either the whole liver homogenate or the 9000 *g* supernatant from warm and cold-exposed groups.

Relation of sleeping time to metabolism in vitro. Although the sleep time of the control rats does decrease convincingly between the first and third weeks of the study when the rats are 37–51 days of age, the cold-exposed groups do not show this age-related

TABLE 2. HEXOBARBITAL DOSE–RESPONSE AT 0 AND 3 DAYS OF EXPOSURE TO 5°*

Hexobarbital (mg/kg)	Sleeping time (min ± S.E.)			
	Nov. 21, 1966		Apr. 24, 1967	
	Warm (0 day)	Cold (3 days)	Warm (0 day)	Cold (3 days)
48	0·8 ± 0·58 (5)	3·6 ± 1·41 (5)		
69	15·0 ± 3·16 (5)	13·0 ± 0·63 (5)		
100	29·2 ± 2·83 (5)	32·0 ± 9·33 (5)	37·1 ± 3·38 (7)	50·4 ± 5·12 (7)†
144	62·8 ± 5·57 (5)	73·6 ± 12·25 (5)	50·9 ± 10·30 (7)‡	103·4 ± 13·6 (5)‡§
208			100·7 ± 15·80 (7)‡	278·0 ± — (2)‡

* Male, Wistar 36-day-old rats injected i.p. with appropriate doses of hexobarbital in the volume of 5 ml/kg. Number of rats is in parentheses.

† Significantly different from "Warm" at P = 0·05.

§ Significantly different from "Warm" at P = 0·01.

‡

	Mortality	
Dose	*Warm*	*Cold*
144 mg/kg	1/8	3/8
208 mg/kg	1/8	5/7

change. Rather, their sleeping time becomes greater than their age-matched controls within 3–7 days of exposure and, in spite of considerable fluctuations, remains higher for the entire 7 weeks. The patterns of the metabolism of hexobarbital *in vitro* correspond with the sleeping time results for the controls, i.e. the rate of metabolism of controls increases most markedly between 1 and 3 weeks while that of the cold-exposed groups remains at initial levels throughout.

Brain concentration of hexobarbital. All groups studied awakened at similar brain concentrations of hexobarbital, irrespective of dose or environmental conditioning

TABLE 3. BRAIN CONCENTRATIONS OF HEXOBARBITAL UPON AWAKENING OR UPON DEATH*

Hexobarbital (mg/kg)	Group	Awakening		Death	
		Brain concn (μg/g ± S.E.)	Sleep time (min ± S.E.)	Brain concn (μg/g ± S.E.)	Time to death (min ± S.E.)
4/3/67					
100	Warm† (0 day)	38·4 ± 1·20	23·3 ± 4·65 (7)		
	Cold (3 days)	34·7 ± 1·30	45·6 ± 3·67 (7)		
100	Warm‡ (0 day)	38·3 ± 1·30	36·0 ± 9·14 (6)		
	Cold (3 days)	37·1 ± 4·68	60·5 ± 8·84 (4)	73·2	6·0 (1)
4/24/67					
100	Warm‡ (0 day)	39·0 ± 1·55	37·1 ± 3·38 (7)		
	Cold (3 days)	36·1 ± 1·07	50·4 ± 5·12 (7)		
144	Warm‡ (0 day)	37·9 ± 1·04	50·9 ± 10·30 (7)	108·6	8·0 (1)
	Cold (3 days)	35·0 ± 2·69	103·4 ± 13·56 (5)	121·0 ± 11·0	4·7 ± 0·66 (3)
208	Warm‡ (0 day)	39·1 ± 1·92	100·7 ± 15·75 (7)	203·9	26·0 (1)
	Cold (3 days)	37·6 ± —	278 (2)	223·1 ± 2·16	24·0 ± 3·73 (5)

Male, Wistar rats injected i.p. with doses of hexobarbital sodium noted in table. Rats exposed to 5° environment for 0 (Warm) or 3 (Cold) days. Number of rats is in parentheses.

† Rats sprayed with pyrethrin-containing insecticide for 3 days prior to 5° exposure (Cold) or for 6 days in Warm (25°) exposure.

‡ Rats not sprayed with insecticide.

(Table 3). This would indicate an unchanged sensitivity to the hypnotic action of the drug. At time of death, brain concentration of the drug was approximately the same for both the cold-exposed and warm groups and varied with the administered dose.

Seasonal effect on hypnotic action. When several groups of rats were observed in the fall of the year, prolonged sleeping times could not be obtained after a 3-day cold exposure (Table 4), but in April of the following year the effect was again observed. These observations were extended by dose–response curves obtained in November and in April (Table 2). There was no significant difference between the warm and cold-exposed groups in November, but there was a significant difference in April. These results suggest either a seasonal effect or differences in the several batches of animals (e.g. we cannot "control" exactly what the animal suppliers do to them with regard to insecticides, feeding, handling etc.). The differences are not very large and in one instance they are reversed (Table 4, 11/4/66).

TABLE 4. SEASONAL EFFECT ON HEXOBARBITAL SLEEPING TIME AFTER COLD EXPOSURE

Date	Sleeping time (min. ± S.E.) 0 Day (cold)*	3 Days (cold)
4/1/66	25·6 ± 2·26 (10)	38·0 ± 5·06 (5)†
4/26/66	23·3 ± 1·85 (9)	42·6 ± 5·95 (5)‡
11/4/66	40·6 ± 2·60 (5)	32·0 ± 2·60 (4)†
11/21/66	29·2 ± 2·83 (5)	32·0 ± 9·33 (5)
4/3/67	36·0 ± 9·14 (6)	60·5 ± 8·84 (4)†
4/24/67	37·1 ± 3·38 (7)	50·4 ± 5·12 (7)†

* Male, Wistar rats, 30–36 days of age, injected with 100 mg/kg hexobarbital sodium after 0 or 3 days, of exposure to 5°. Number of rats is in parentheses.

† Significantly different from 0 day cold group at $P < 0{\cdot}05$.

‡ Significantly different from 0 day cold group at $P < 0{\cdot}01$.

DISCUSSION

The most important finding of these experiments is that rats exposed to 5° for at least 3 days and for as long as 7 weeks sleep longer than their age-matched 25° controls when given hexobarbital. This increase in sleep time does not correspond with a diminished metabolism, *in vitro* since the rate of metabolism by the liver of the cold-exposed rats remains essentially constant for the entire 7 weeks. This contrasts with the pattern seen over a corresponding time interval for the control animals, in which the sleep times decreased markedly between 1 and 3 weeks and this corresponded with marked increases in metabolism *in vitro* during this period.

An age-related change in sleep time occurs in the control rats; minimum sleep times occur when the rats are 44–58 days of age (2–4 weeks at 25°). These results with male, Wistar rats differ somewhat from those of Kato *et al.*[14] in which female Sprague–Dawley rats were used. In their work most of the drugs tested, including pentobarbital, showed a minimum duration of response when the rats were 30–100 days of age. The cold-exposed rats do not show this change with chronologic age.

Although the animals of the two exposure regimens are age-matched, they are not

weight-matched, as is seen in the data of Fig. 1, and their body composition differs. Fig. 1 shows that the liver comprises a greater percentage of the body weight in the cold-exposed rat; body fat is decreased more rapidly than body weight in the first 48 hr of cold exposure;[15, 16] muscle mass is decreased and many other changes in body composition occur.[17] Although metabolism is considered to be the most important factor in the termination of action of hexobarbital, the influence of tissue distribution cannot be entirely ruled out. When body compositions vary markedly, as they do under these two environmental conditions, the role played by this factor may be of greater importance. With respect to the laying down of depot fat, the cold-exposed animal presents the composition of a younger animal.

In the relatively few reports available, varying changes in hexobarbital metabolism after cold exposure have been reported; a decreased rate of hexobarbital metabolism after 1–2 days of exposure to 4°;[2] an increased rate of metabolism after 4 days of exposure to 4°;[3] no change in the rate of metabolism after 2–3 days of exposure to 5° (present paper). The long-term phase of cold exposure has not been reported. A report appearing after this paper had been submitted[18] shows varying time courses for microsomal enzyme activity in the hydroxylation of aniline and the *N*-dealkylation of ethylmorphine, but both appear to be elevated from control by 1 day and remain elevated for the 7 days investigated. However, the above paper does not deal with pharmacological effects. The data presented in the present report do not attempt to resolve the differences seen by the various investigators, but the few experiments performed in the fall of the year, where sleeping times were either slightly decreased or unchanged after 3 days of cold exposure, in contrast to the increase in sleeping time seen in the experiments performed in the spring of the year, suggest that a seasonal effect may be at least one contributing factor. In another form of stress, ligature trauma, Bousquet *et al.* (personal communication) noted a seasonal effect on adrenal ascorbic acid depletion in rats. Outdoor rats are known to undergo seasonal changes in pelt thickness and in oxygen consumption[19] and it may not be unreasonable to see a seasonal effect to temperature stress in laboratory raised rats.

No significant change in the brain level of barbiturate at which the rats awaken is apparent between the groups exposed to 25° or 5° for 3 days. Regardless of the dose of drug or the environmental regimen, all groups awaken at a brain level of 35–39 μg/g. This would suggest that no marked difference in brain sensitivity exists between groups, even though the cold-exposed groups do exhibit longer sleeping times and a greater toxicity to the drug.

Although we do not think the control animals were exposed to conditions which would cause enzyme induction while the cold-exposed animals were protected from such conditions, we cannot completely exclude such a possibility. All animals were housed singly and in wire-mesh cages and were thus protected from the effects of aggregation and of cedar wood bedding which have recently been shown to be inducing agents.[20] The effect of the pyrethrin insecticide, which was inadvertently used in the warm room during part of one experiment, was tested and found not to have caused a difference. The lighting schedules in the warm and cold rooms were not strictly comparable; the warm room had constant light whereas the cold room had light during the working day from about 8 a.m. to 5 p.m., but not on Sundays. Two groups of warm-room rats were checked for the effect of constant versus cyclic light periods over a 1-week period and no significant difference in hexobarbital

sleeping times was seen. In the warm room chickens were also present, whereas in the cold room only rats were present. The same food was presented to both groups, but cold-exposed animals ate larger quantities.

Our rates of metabolism *in vitro* are relatively high when compared with some of the older literature in which incubation periods of 1–1½ hr were used, but it has recently been shown[21] that the reaction rate is not constant in the rat for such long incubation times. Our rates are comparable with those of Gillette[3] (3·94–4·2 μmole/g/30 min). Much variation exists in the literature for absolute rates of hexobarbital metabolism *in vitro*.

There is also the distinct possibility that the rates of hepatic metabolism *in vitro* do not accurately reflect the rates *in vivo* during part or all of the exposure periods. Without greatly expanded cold-room facilities, we are not equipped to check this possibility.

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