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48. Eigo Takabatake: Metabolism of Drugs. XI.* The Relationship between Hypnotic Activity and Metabolism of Ethylhexabital. (2). The Effect of Sex Hormones.

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In the preceding paper,[1] it was shown that the sex difference in the duration of hypnosis produced by ethylhexabital (EHB) in the adult rat was due to the difference in the EHB-metabolizing activity of the liver. The effect of sex hormones on barbiturate anesthesia has been studied by several investigators[2] mainly on the duration of hypnosis or the lethal dose. On the other hand, it is known that the oxygen uptake of the brain is inhibited by both steroids including testosterone or estradiol and barbiturates, although the site of their inhibitory action is different. In the present paper, the effect of sex hormones on EHB hypnosis is described in connection with both the EHB-metabolizing activity of the liver and sensitivity of the brain to EHB.

Methods

The rat was castrated under slight ether anesthesia.

Hormone administration: Aqueous suspension of testosterone (5 mg./cc.), aqueous suspension of estradiol monobenzoate (0.2 mg./cc.), or oil solution of progesterone (10 mg./cc.) was subcutaneously injected.

The determination of duration of hypnosis and the *in vitro* metabolism were performed as described in the previous papers.[1,3] In some cases the EHB-metabolizing activity was estimated only from the quantity of unchanged EHB by the following procedure. After 250 mg. of liver slices was incubated with 250 γ of EHB in 4 cc. of Krebs-Ringer phosphate buffer (pH 7.4) containing 0.2% glucose (KRPG) for 3 hrs. at 37.5°, the content was extracted with two 15-cc. portions of petroleum ether containing 2.5% of isoamyl alcohol. This solvent extracted only EHB and not 3-keto-EHB. EHB in organic phase was reëxtracted with 5 cc. of borate buffer (pH 11) and the quantity of EHB was calculated from the optical density at 239 mμ.

Determination of Respiratory Rate of the Brain: The rat was decapitated and the homogenization of its brain in a volume of KRPG sufficient to make a 10% preparation was carried out at 0°, in the apparatus of Potter and Elvehjem. Two cc. of the homogenate and 0.8 cc. of KRPG were added into the flask. EHB was placed in the side arm as 0.2 cc. of $3\times10^{-2}M$ solution and tipped in at 0 or 60 mins. The gas phase was oxygen and 0.2 cc. of 20% KOH was placed on a filter paper in the center well. The flasks were placed in a bath at 37.5° and readings were taken every 10 mins. for 120 mins.

Determination of the Blood Level of EHB and 3-Keto-EHB: One-half cc. of blood was drawn from the heart into a syringe containing 0.1 cc. of 3.8% sodium citrate solution and was poured into a glass-stoppered tube. After the syringe was washed twice with 0.5 cc. each of citrate solution, the combined content was made acidic with one drop of conc. HCl and extracted twice with 12.5 cc. each of ether by shaking for 20 mins. Combined ether extract was dried over 1 g. of Na_2SO_4, decolorized with 0.1 g. of activated charcoal, and then filtered through a sintered-glass filter. Twenty cc. of the filtrate was pipetted into a small flask and evaporated to dryness. The residue was dissolved in 0.4 cc. of MeOH, and 0.2 cc. of MeOH solution was subjected to paper chromatography. The subsequent procedures were just as in the case of the urine[4] or the *in vitro* metabolism[3] already described.

Results

I. Effect of Castration and Hormone Administration in Adult Male Rats

Adult male rats were divided into three groups: A-Group, intact control; B-group, castrated

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1) Part X. E. Takabatake: This Bulletin, **5**, 260(1957).
2) H. G. O. Holck, D. R. Mathieson: J. Am. Pharm. Assoc., **33**, 174(1944).
3) E. Takabatake: J. Pharm. Assoc. Japan, **76**, 511(1956).
4) H. Tsukamoto, E. Takabatake, T. Ariyoshi: This Bulletin, **3**, 459(1955).

4 weeks before the determination, and C-group castrated and subcutaneously injected with 0.75 mg. of testosteorne daily for the final 1 week. The experimental data presented in Table I show that the castration lengthened the duration of EHB hypnosis, while the testosterone administration shortened the duration prolonged by castration nearly to a normal level. The blood level of EHB in A-group which had already recovered from hypnosis was lower at 1 hr. later than that of other groups. Two hrs. later, the difference between those of C- and A-groups was not significant but that of B-group was significantly higher than that of A-group. The blood level of 3-keto-EHB was reciprocal to the EHB level and even at 2 hrs. later, that of B-group was lower than that of other groups. Table II shows the EHB-metabolizing activities of liver slices obtained from the same treated rats. The data regarding the oxygen uptake of the brain are presented in Table III. Estradiol monobenzoate was administered for 1 week in a daily dose of 0.04 mg. per rat. It was apparent that the testosterone treatment restored the activity lowered by castration but that estradiol lowered the activity of intact or castrated rats. However, the respiratory rate of the brain and the percentage of inhibition by EHB were not influenced by any treatment.

Table I. Effect of Castration and Hormone Administration on the Duration of EHB Hypnosis and the Blood Level of EHB and 3-Keto-EHB in Adult Male Rats

Group	Treatment	No. of rats	Body wt. g.	Duration* min.	Blood level (mg./L.) EHB 1 hr.	EHB 2 hrs.	3-Keto-EHB 1 hr.	3-Keto-EHB 2 hrs.
A	Intact control	4	214	62	80	46	29	30
B	Castration	4	192	231	108	81	17	21
C	Castration+testosterone	3	196	107	100	69	19	29

* EHB (100 mg./kg.) was intraperitoneally injected.

Table II. Effect of Castration and Hormone Administration on the EHB-Metabolizing Activity of Liver Slices of Adult Male Rats

Group	Treatment	No. of rats	Body wt. g.	Sv+P* wt. mg.	EHB remained γ	3-Keto-EHB formed γ
A	Intact control	7	207	784	135	257
B	Castration (C)	9	191	60	246	175
C	(C)+testosterone	9	192	707	181	234
D	(C)+estradiol	5	170		257	140

Significance of differences ($p<0.05$): A-B, B-C, C-D, D-A

* Weight of seminal vesicle and prostate

Table III. Effect of Castration and Hormone Administration on the Oxygen Uptake of Brain of Adult Male Rats

Group	Treatment	No. of rats	Body wt. g.	Sv+P* wt. mg.	Metabolized EHB** %	Q_{O_2} during 1st 60 mins. no EHB	EHB added at 0 min.	Inhibition by EHB %	Q_{O_2} during 2nd 60 mins. no EHB	EHB added at 60 min.	Inhibition by EHB %
A	Intact control (I)	5	188	655	55.0	5.41	3.36	38.0	4.49	2.32	48.3
B	Castration (C)	5	184	110	26.1	5.24	3.27	37.6	4.69	2.30	51.0
C	(C)+testosterone	5	200	690	54.1	5.62	3.51	37.5	5.15	2.63	48.9
D	(I)+estradiol	5	177	530	40.2	5.16	3.06	40.7	4.62	2.26	51.1

* Weight of seminal vesicle and prostate

** Estimated only from the quantity of remaining EHB

II. Effect of Ovariectomy and Hormone Administration in Adult Female Rats

Adult female rats were divided into six groups, A-group was intact control and the others were ovariectomized 4 weeks before the assay. Hormone was subcutaneously injected for 1 week prior to the assay and the daily dose per rat for each group was as follows: 0.75 mg. of testosterone for C-group, 0.03 mg. of estradiol monobenzoate for D-group, 0.75 mg. of progesterone for E, and 0.03 mg. of estradiol monobenzoate plus 0.75 mg. of progesterone for F-group. The results are presented in Tables IV and V. Ovariectomy did not alter the duration of EHB hypnosis but only testosterone shortened it significantly. Estradiol lengthened the duration but the difference between the groups D and B was not significant because of the marked variation. The tendency to shorten the duration by progesterone was observed. The rats of C-group, ovariectomized and treated with testosterone,

indicated the lower blood level of EHB and the higher level of 3-keto-EHB than the other groups. Testosterone significantly enhanced the EHB metabolizing activity of liver slices of ovariectomized rat, while the effect of ovariectomy or estradiol treatment was not significantly recognized. Subsequent experiment was carried out to ascertain whether or not the susceptibility of the liver to estrogen had been reduced after maturation.

Table IV. Effect of Ovariectomy and Hormone Administration on the Duration of EHB Hypnosis and Blood Levels of EHB and 3-Keto-EHB in Adult Female Rats

Group	Treatment	No. of rats	Body wt. g.	Duration* min.	Blood level 2 hrs. later	
					EHB mg./L.	3-Keto-EHB mg./L.
A	Intact control	4	139	261	99	24
B	Ovariectomy (O)	5	180	263	91	15
C	(O)+testosterone	5	188	139	77	27
D	(O)+estradiol	4	166	310	88	19
E	(O)+progesterone	5	170	221	90	15
F	(O)+estradiol+progesterone	5	146	231	78	17

Significance of difference in duration ($p<0.05$): C-A, B, D, E, F, D-E

* EHB (100 mg./kg.) was intraperitoneally injected.

Table V. Effect of Ovariectomy and Hormone Administration on the EHB-Metabolizing Activity of Liver of Adult Female Rats

Group	Treatment	No. of rats	Body wt. g.	Remaining EHB γ	3-Keto-EHB formed γ
A	Intact control	5	140	349	181
B	Ovariectomy (O)	5	176	319	141
C	(O)+testostesone	5	170	267	191
D	(O)+estradiol	5	156	359	115
E	(O)+progesterone	5	173	336	131
F	(O)+estradiol+progesterone	4	155	370	110

Significance of difference ($p<0.05$): C-A, B, D, E

III. Effect of Ovariectomy before Maturation and Hormone Administration in Female Rats

The following four groups of female rats were studied: A-group of intact control, B-group ovariectomized before opening of vagina (body wt. about 50 g.), C-group ovariectomized at the same time as B but injected 12 times during 2 weeks prior to the assay with 0.04 mg./day of estradiol monobenzoate per rat, per day, and D-group treated the same as C-group except receiving 0.02 mg. of the hormone. The experimental results are presented in Table VI. Ovariectomy shortened the duration of EHB hypnosis and enhanced the EHB-metabolizing activity of the liver, while estradiol lengthened the duration and reduced the metabolizing activity of the liver. The effect of high dose of estradiol was more evident in the uterine weight but was equal to that of lower dose on the EHB-metabolizing activity of the liver. The data presented in Table VII show that both the respiratory rate of the brain and sensitivity to EHB were not affected by any treatment. The rats of D-group in this experiment were injected with 0.75 mg. of testosterone daily for 1 week.

Table VI. Effect of Ovariectomy before Maturation and Hormone Administration on the EHB-metabolizing Activity of Liver Slices of Female Rats

Group	Treatment	No. of rats	Body wt. g.	Duration min.	No. of rats	Body wt. g.	Uterine wt. mg.	Remaining EHB γ	3-Keto-EHB formed γ
A	Control	5	125	248	5	127	475	249	161
B	Ovariectomy (O)	4	132	189	5	144	42	210	191
C	(O)+estradiol (high)	4	107	261	5	117	708	289	138
D	(O)+estradiol (low)	4	107	233	5	121	421	300	138
Significance of difference ($p<0.05$):		B-A, D						B-C, D	

IV. Antagonistic Action of Estradiol to Testosterone

1) Effect in Castrated Rats: The castrated male or female rats were assayed 4 weeks after the operation. A part of rats was injected with 0.75 mg. of testosterone and another part with both 0.75 mg. of testosterone and 0.06 mg. of estradiol monobenzoate daily for 1 week prior to the *in vitro* experiment. The results are shown in Table VIII. Estradiol significantly reduced the accelerating effect of testosterone on the EHB-metabolizing activity of the liver.

Table VII. Effect of Ovariectomy and Hormone Administration on the Oxygen Uptake of Brain of Female Rats

Group	Treatment	No. of rats	Body wt. (g.)	Metabolized EHB* (%)	Q_{O_2} during 1st 60 mins.			Q_{O_2} during 2nd 60 mins.		
					No EHB	EHB added at 0 min.	Inhibition by EHB %	No EHB	EHB added at 60 min.	Inhibition by EHB %
A	Intact control (I)	5	155	24.5	5.88	3.60	38.8	5.30	2.42	54.3
B	Ovariectomy (O)	5	151	36.6	5.45	3.41	37.5	5.11	2.61	48.9
C	(O)+estradiol	5	138	29.1	5.56	3.32	40.3	4.98	2.57	48.3
D	(I)+testosterone	5	149	36.7	5.79	3.77	35.0	5.01	2.58	48.7

* Estimated only from the quantity of remaining EHB.

Table VIII. Antagonistic Effect between Testosterone and Estradiol on the EHB-Metabolizing Activity of the Liver of Castrated Rats

Treatment	Male				Female			
	No. of rats	Body wt. (g.)	EHB remained	3-Keto-EHB formed	No. of rats	Body wt. (g.)	EHB remained	3-Keto-EHB formed
Testosterone	5	151	226	225	3	129	206	232
Testosterone +estradiol	5	146	305	185	**5**	116	302	169
Significance of difference, p			$<.01$	$<.01$			$<.01$	$<.01$

2) Experiments in Normal Rats: Rats of both sexes were respectively divided into three groups: 1st group was the normal control, 2nd group injected with 0.75 mg. of testosterone daily for 1 week, and 3rd group injected with 0.04 mg. of estradiol monobenzoate daily for 1 week. Fig. 1 shows the relative EHB-metabolizing activity of each group compared with that of female normal control. Estradiol significantly reduced the EHB-metabolizing activity of male rat liver and the accelerating effect of testosterone on this activity was less in the female than in the male. These facts seem to suggest that estradiol antagonizes the effect of testosterone rather than lowering metabolizing activity of the liver

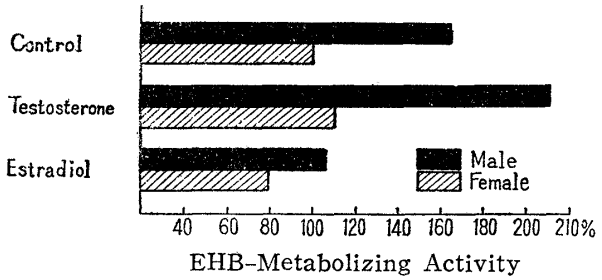

Fig. 1.
Effect of Hormone Administration on the EHB-Metabolizing Activity in Normal Rats (The activity of normal female rat was taken 100% as standard)

V. Effect of Testosterone on the Metabolism of EHB by Mouse Liver

After administration of 0.15 mg. of testosterone daily for 1 week to the castrated male mouse, the amount of 3-keto-EHB formed in liver slices *in vitro* was determined. The data were 118 γ in the castrated control and 103 γ in the testosterone-treated castrate (5 mice each). In contrast to rats, no accelerating effect of this hormone was found in mice though the accessary organs were stimulated by this treatment.

Discussion

It has been known that the male rat is more resistant than the female to barbiturates such as Amytal,[5] pentobarbital,[2,5~11] Pernoston,[5] Evipal,[5,12] and EHB.[1,5] These drugs

5) H. G. O. Holck, M. A. Kanân, L. M. Mills, S. L. Smith: J. Pharmacol. Exptl. Therap., **60**, 323 (1937).
6) C. R. Cameron: Proc. Roy. Soc. Med., **32**, 309(1939).
7) C. R. Cameron, G. H. Cooray, S. N. DeSaram: J. Pathol. Bacteriol., **60**, 239(1948).
8) W. M. Moir: J. Pharmacol. Exptl. Therap., **59**, 68(1937).
9) H. G. O. Holck, D. R. Mathieson, E. L. Smith, L. D. Fink: J. Am. Pharm. Assoc., Sci. ed., **31**, 116(1942).

belong to barbiturates of short duration and are rapidly biotransformed in the animal body. On the other hand, no sex difference was found in barbital,[5] a long-acting barbiturate, which was not metabolized at all. It has been considered from these facts that sex difference in the duration of barbiturate hypnosis might be due to the difference in metabolizing activity of the liver which was affected by sex hormones. The duration of barbiturate hypnosis is said to be prolonged by castration in the male and to be shortened by testosterone.[2,7~11] Past reports on the effect of ovariectomy in the female rat were conflicting, this operation was said to increase susceptibility[6] or resistance[10,11] to pentobarbital, or to have no influence.[5,13] It was found that estradiol reduced the rate of pentobarbital metabolism in the liver[12] and lengthened the duration of hypnosis by this drug.[10,11]

Based on the formation rate of 3-keto-EHB, a pharmacologically inactive metabolite of EHB, in liver slices *in vitro*, it was apparent as shown in this paper that the sex difference in duration of EHB hypnosis was due to the difference in the metabolic rate of EHB by the liver which is regulated by sex hormones. The accelerating effect of testosterone on the EHB-metabolizing activity was observed in normal or castrated rats of both sexes and could adequately explain the higher resistance of adult male rats to EHB. The EHB-metabolizing activity of the liver was accelerated by ovariectomy before maturation but not after it. The lowering effect of estradiol was not found in the adult female which had once been influenced by endogeneous estrogen but more evidently found when estradiol was administered with testosterone in the castrated rats or in normal male which was controlled by endogeneous androgen. Estradiol was considered to antagonize the accelerating effect of testosterone rather than influence the liver itself. It was also reported[5] that neither ovariectomy of adult female rats nor oestrus influenced the response to Evipal.

There seems little doubt that one of the possible ways in which barbiturates and steroids produce their anesthetic effect is by interference of oxidative processes of the cell.[14,15] According to Eisenberg, *et al.*,[16] castration performed at 30 days produced a marked increase in the rate of glucose utilization and administration of testosterone in 1 mg./day dose for 30 days *in vivo* prevented this increase. The addition of testosterone *in vitro* further suppressed the oxygen uptake but the inhibition was far less in the castrates than in either the castrates treated with testosterone *in vivo* or the normal rat. However, Gordan[17] reported that castration, when performed upon older male rats, did not result in an elevation of oxygen consumption of the brain. It is widely known that barbiturates inhibit the *in vitro* respiration of brain in the presence of glucose.[14] Under the experimental conditions mentioned above, the EHB-metabolizing activity of the liver was influenced by castration and hormone administrations, but the oxygen uptake of the brain and inhibitory effect of EHB were not affected by the same treatment in either sexes. It is concluded that the influence of sex hormones on the duration of EHB hypnosis is mainly due to their effect on the liver rather than on sensitivity of the brain.

The sex difference in the metabolism of EHB was found only in the rat as shown in the preceding paper[1] and testosterone did not affect the EHB-metabolizing activity of

10) M. Crevier, A. D'Iorio, E. Robillard; Rev. can. biol., **9**, 336(1950).
11) E. Robillard, A. D'Iorio, J. Pellerin : Union med. Canada, **83**, 853(1954).
12) B. B. Brodie : J. Pharm. Pharmacol., 8, 1(1955).
13) L. Buchel : Anesthésie et analgésie, **11**, 228(1954).
14) J. H. Quastel : Physiol. Rev., **19**, 135(1939).
15) F. E. Hunter, O. H. Lowry : Pharmacol. Rev., 8, 89(1956).
16) E. Eisenberg, G. S. Gordan, H. W. Elliott : Science, **109**, 337(1949).
17) G. S. Gordan, R. C. Bentinck, E. Eisenberg : Ann. N. Y. Acad. Sci., **54**, 575(1951).

the liver of castrated male mouse. Brodie[12] reported that estradiol and testosterone could not induce any appreciable change in the detoxication rate of Evipal in normal mice and rabbits.

Mechanisms by which the sex hormone affects the liver are not yet known. Holck, *et al.*[9] suggested a parallel relationship between the liver glycogen content and resistance to pentobarbital hypnosis. Booker, *et al.*[18] said that the metabolism of thiopental was accelerated with the increase of liver glycogen. However, it seemed probable that the main factor regulating the duration of thiopental hypnosis was the depositing rate of drug in the body fat. No clear-cut relation between liver glycogen content and the EHB-metabolizing activity of the liver was observed in our preliminary experiment. Starvation lowered the amount of liver glycogen but did not influence the duration of EHB metabolism *in vitro* or Evipal hypnosis.[5] Cameron[6] suggested that the liver inactivated sex hormones and that such hormones might be utilized as co-factors in liver detoxication. N-Demethylation of morphine[19] and metabolism of Evipal[12] with liver microsome preparation were said to be accelerated by testosterone and to be reduced by estradiol. It is assumed that the pathway of EHB to 3-keto-EHB also proceeds by the similar enzyme systems which would be affected by sex hormones.

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Summary

The effect of sex hormones on EHB hypnosis was investigated from both the EHB-metabolizing activity of the liver and sensitivity of the brain to EHB.

In the male rat, castration lowered EHB-metabolizing activity of the liver and testosterone treatment accelerated this lowered activity. Estradiol did not alter the activity of castrated rat liver but lowered that of normal rat.

In the female rat, the EHB-metabolizing activity of the liver was accelerated by ovariectomy before but not after maturation. Estradiol antagonized the accelerating effect of testosterone on the metabolism of EHB in the liver.

In both sexes, the respiratory rate and sensitivity of the brain to EHB were not influenced by any treatment.

In the castrated mouse, testosterone did not influence the EHB-metabolizing activity of the liver.

It was concluded that the influence of sex hormones on the duration of EHB hypnosis was mainly due to their effect on EHB-metabolizing activity of the liver and not on sensitivity of the brain to EHB.

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18) W. M. Booker: Am. J. Physiol., **170**, 168(1952).
19) J. Axelrod: J. Pharmacol. Exptl. Therap., **117**, 322(1956).