UDC 615.782-092.21

152. Eigo Takabatake and Toshihiko Ariyoshi : Biochemical Studies on the Drug Metabolism. I. Some Factors Affecting on the Metabolism of Cyclobarbital.

(*Pharmaceutical Faculty, University of Nagasaki**1)

It has been reported[1~3] that the pretreatment of rats with various drugs including barbiturates and polycyclic hydrocarbons stimulated the activity of drug-metabolizing enzymes localized in the microsomes of rat liver. Sex difference[4,5] in these enzyme has been demonstrated. In the course of the authors' studies,[6] it was found that cyclobarbital(EHB)*2-metabolizing activity of rat liver slices was enhanced by the treatment with testosterone or EHB. EHB is metabolized to 3-keto-EHB(5-ethyl-5-(3-oxo-1-cyclohexenyl)barbituric acid)[7] and 3-OH-EHB(5-ethyl-5-(3-hydroxy-1-cyclohexenyl)barbituric acid).[8] It is not clear whether hydroxylation or ketone oxidation occurs first, but these two metabolites are interconvertible just similar to metabolites of hexobarbital(5-(1-cyclohexenyl)-3,5-dimethylbarbituric acid).

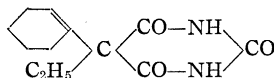
The work presented here was attempted to determine the site which was stimulated by the pretreatment with testosterone or EHB. Furthermore, it was examined whether the effect of EHB was mediated by its metabolites or not.

Methods

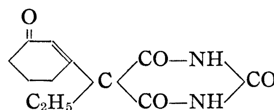
The animals used were Wistar rats and fed Oriental rat diet-MN for 1 week prior to the assay.

Aqueous suspensoin of testosterone (1 mg./0.1 cc.) was subcutaneously injected once daily for 7 days. EHB was prepared from its calcium salt and recrystallized from waier. 3-OH-EHB[8] and 3-keto-EHB[7] were chemically prepared from EHB. These compounds were intraperitoneally injected in single dose of 100 mg./kg. of body weight.

Cyclobarbital (EHB) : 5-ethyl-5-(1-cyclohexenyl)barbituric acid



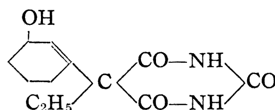
3-Keto-EHB : 5-ethyl-5-(3-oxo-1-cyclohexenyl)barbituric acid



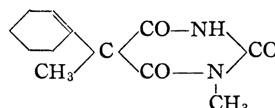
*1 Showa-machi, Nagasaki (高畠英伍, 有吉敏彦).
*2 The abbreviation of EHB would be used for 5-ethyl-5-(1-cyclohexenyl)barbituric acid in this series although its official name has been changed from Ethylhexabital to Cyclobarbital in J.P. VII.

1) A.H. Conney, J.R. Gillette, J.K. Inscoe, E.R. Trams, H.S. Posner : Science, **130**, 1478 (1959).
2) A.H. Conney, C. Davison, R. Gastel, J.J. Burns : J. Pharmacol. Exptl. Therap., **130**, 1 (1960).
3) A.H. Conney, I.A. Michaelson, J.J. Burns : *Ibid.*, **132**, 202 (1961).
4) B.B. Brodie : J. Pharm. and Pharmacol., **8**, 1 (1956).
5) B.B. Brodie, J.R. Gillette, B.N. LaDu : Ann. Rev. Biochem., **27**, 427 (1958).
6) E. Takabatake : This Bulletin, **5**, 260, 266 (1957).
7) H. Tsukamoto, E. Takabatake, H. Yoshimura : *Ibid.*, **2**, 201 (1954).
8) H. Tsukamoto, Y. Kuroiwa : *Ibid.*, **7**, 731(1959).

3-OH-EHB : 5-ethyl-5-(3-hydroxy-1-cyclohexenyl)barbituric acid



Hexobarbital : 5-(1-cyclohexenyl)-3,5-dimethylbarbituric acid



Rats pretreated with drugs were sacrificed by decapitation 24 hours later, and the liver was removed and homogenized with 2 volume of 0.1*M* phosphate buffer, pH 7.4, in cold. The homogenates were centrifuged for 20 minute at 9000×g. at 0°, and the supernatants were assayed for the activities to metabolize EHB, 3-OH-EHB, and 3-keto-EHB, respectively. An incubation mixture contained 2 cc. of 9,000 g. supernatant, 50 μmole of nicotinamide, 25 μmole of magnesium chloride, and 0.5 cc. of substrate solution of water (blank) to make final volume to 4 cc. The mixture was incubated for 1.5 hour at 37° in Warburg apparatus. After incubation, the mixture was heated for 5 minutes on a boiling water bath, cooled, adjusted to pH 5.0 with conc. hydrochloricacid, and then continuously extracted with ether for 10 hours. The ether extract was treated with activated charcoal, dried over sodium sulfate, and filtered. The residue obtained by evaporation of ether was dissolved in 0.5 cc. of methanol and 0.05 cc. of this solution was chromatographed on paper treated previously with borate buffer of pH 10.0. The developing solvent was butanol saturated with borate buffer of pH 11.0 and ascending procedure was employed. The portions of paper corresponding to EHB, 3-OH-EHB, and 3-keto-EHB were respectively cut off and extracted with 5 cc. of borate buffer of pH 11.0. The quantity of barbiturates was calculated from the absorbancy at 239 mμ. A blank run in parallel was carried out and its value was subtracted from sample run.

Results

As shown in Table I, the liver preparation of intact male rats markedly metabolized EHB, thus the amount of EHB remained was only about 28% of added EHB and 57% was converted to 3-OH-EHB. The liver of females could metabolize only about

TABLE I.[a] The Effects of Sex, Testosterone, EHB, and EHB-Metabolites on the EHB-Metabolizing Activity of the Rat Liver

Sex	Pretreatment	No. of rats	Remained EHB (%)	Formed 3-OH-EHB (%)	Formed 3-keto-EHB (%)
M	None	8	28.4±8.7	56.8±13.8	3.5±1.6
F	None	6	60.8±5.5	20.1±3.3	3.2±1.7
F	Testosterone				
	1 mg.×7	5	47.8±2.8	31.3±5.6	2.7±1.8
	2 mg.×7	2	47.1	40.6	7.4
F	None	14	55.1±3.7	16.2±2.4	5.1±0.6
F	EHB	8	34.3±5.2	33.4±5.5	10.0±2.0
F	3-OH-EHB	9	41.2±4.8	39.9±5.2	0.8±0.2
F	3-keto-EHB	6	58.4±3.8	9.7±0.9	2.1±1.2

a) In Tables, M and F represent male and female respectively, and the average ± standard error are given.

9) H. Tsukamoto, S. Toki, K. Kaneda : *Ibid.*, **7**, 651 (1959).

40% of added EHB and the amount of 3-OH-EHB formed was 20%. However, no difference was shown in the amount of formation of 3-keto-EHB. The activity of liver female to metabolize EHB was increased by repeated administration of testosterone.

The pretreatment with EHB accelerated the metabolism of EHB in female rats, and both the formation of 3-OH-EHB and that of 3-keto-EHB were stimulated. The pretreatment with 3-OH-EHB also accelerated the metabolism of EHB, and the formation of 3-OH-EHB was markedly enhanced but that of 3-keto-EHB was depressed. The pretreatment with 3-keto-EHB did not show any such effect.

Table II. The Effects of Sex, Testosterone, EHB, and 3-OH-EHB on the *in vitro* Conversion of 3-OH-EHB to 3-Keto-EHB by Rat Liver

Sex	Pretreatment	No. of rats	Remained 3-OH-EHB (%)	Formed 3-keto-EHB (%)
M	None	8	72.6±7.9	19.5±7.4
F	None	7	49.5±4.1	40.8±5.4
F	Testosterone			
	1 mg.×7	5	62.8±4.8	29.4±5.2
	2 mg.×7	2	69.8	20.1
F	None	7	56.8±3.1	35.1±3.9
F	EHB	8	61.3±4.1	28.6±2.5
F	3-OH-EHB	8	79.1±3.2	6.9±3.3

Table III. The Effects of Sex, Testosterone, EHB and 3-OH-EHB on the *in vitro* Conversion of 3-Keto-EHB to 3-OH-EHB by Rat Liver

Sex	Pretreatment	No. of rats	Remained 3-keto-EHB (%)	Formed 3-OH-EHB (%)
M	None	8	57.6±13.8	35.2±14.9
F	None	7	82.2±5.2	1.5±1.0
F	Testosterone			
	1 mg.×7	5	85.9±5.3	7.7±3.8
	2 mg.×7	2	47.6	34.8
F	None	7	84.4±2.3	1.6±0.9
F	EHB	8	91.6±2.6	4.9±1.8
F	3-OH-EHB	8	28.5±16.9	63.5±13.7

The conversion of 3-OH-EHB to 3-keto-EHB by liver preparations was determined as shown in Table II. This conversion was less in male than female and depressed by pretreatment with testosterone or 3-OH-EHB. On the other hand, as shown in Table III, the conversion of 3-keto-EHB to 3-OH-EHB by the liver of male rat was greater than female. This conversion was accordingly enhanced by the pretreatment with 3-OH-EHB in female rat but not influenced by EHB-pretreatment. These high values of 3-OH-EHB are not contributed by the remaining administered material, because the blank value of such livers were just same as nontreated rat livers.

EHB-metabolizing activity of male rat was decreased by adrenalectomy but recovered by the pretreatment with EHB as shown in Table IV. This indicates that the stimulatory effect of EHB is not mediated by the adrenal.

Table IV. The Effect of EHB on the EHB-Metabolizing Activity of Liver of Adrenalectomized Male Rats

Treatment	No. of rats	Remained EHB (%)	Formed	
			3-OH-EHB (%)	3-keto-EHB (%)
Adrenalectomy[a]	5	72.9±3.2	17.8±3.3	0
Adrenalectomy and EHB	7	56.1±2.5	26.5±3.8	3.3±0.7

a) The assay was done 4 days after of adrenalectomy.

Discussion

Sex difference in EHB-metabolizing activities of rats which previously demonstrated in slices of the liver was recognized in this experiment using 9,000 g. supernatant of liver homogenate. Such a sex difference was especially shown in the formation of 3-OH-EHB, but no difference in that of 3-keto-EHB was shown. The sex difference was found also in the interconversion between 3-OH-EHB and 3-keto-EHB. In previous experiment using liver slices, we were afraid to note that both the metabolites were determined as 3-keto-EHB by the simple chromatographic technique.

The adaptive increases in EHB-metabolizing activity of the liver induced by EHB or its metabolites were examined. As mentioned above, the pretreatment with EHB enhanced both the formation of 3-OH-EHB and that of 3-keto-EHB approximately in same proportion as controls. However, in the case of 3-OH-EHB-pretreatment, the formation of 3-OH-EHB was remarkably increased and that of 3-keto-EHB depressed. The fact that the formation of 3-OH-EHB from EHB is remarkable in male or in 3-OH-EHB-pretreated female could be explained by less conversion of 3-OH-EHB to 3-keto-EHB or more conversion of 3-keto-EHB to 3-OH-EHB. The difference of effect between EHB and 3-OH-EHB suggests that the stimulatory effect of EHB on the EHB-metabolizing activity is not necessarily mediated by its metabolite.

It has been reported that drug-metabolizing activity of liver microsomes was adaptively increased by various drugs. The drug-metabolizing enzyme systems seem to have less substrate specificity. By the pretreatment with 3,4-benzpyrene,[1] however, it was shown that the activities of some enzymes were remarkably stimulated but others were slightly influenced. In the case of EHB, that the formation rates of 3-OH-EHB and 3-keto-EHB were not parallel between some treatments suggests the presence of different enzymes.

On the mechanism of adaptive increases in drug-metabolizing enzymes induced by various drugs, it is considered that the drugs increase the biosynthesis of enzyme-protein.[2] In the preliminary studies*3 in this laboratory, it was demonstrated that protein anabolic steroids such as 19-nortestosterone shortened the duration of hypnosis produced by EHB and accelerated the metabolism of EHB. The stimulatory activity of steroids on the EHB-metabolism was more closely related with their myotropic activity (weight of levator ani) than their androgenic activity (weight of the seminal vesicle and prostate). It is wonder that such sex difference in the metabolism of drugs is found only in rat. It would be like to study the mechanism of stimulation by androgen in detail.

The removal of the adrenal glands did not prevent the stimulary effect of EHB on EHB-metabolism. This result agreed with the report of Conney, *et al.*[3] on chlorcyclizine. It has been known that glucocorticoids stimulated the metabolism of hexobarbital[10] or EHB,*4 but it is considered that the different mechanisms are present in the acceleration of drug metabolism.

It is very interesting that, although both 3-OH- and 3-keto-EHB have no hypnotic activity, one has stimulatory effect and the other has not. According to Wilimowski, *et al.*,[11] some barbiturates possessing hydroxypropyl-side chain enhance the narcotic

*3 Presented at the 21st Meeting of Kyushu Branch, Pharmaceutical Society of Japan, in December, 1960.

*4 Unpublished data.

10) H. Remmer : Arch. exper. Path. Pharmakol., **233**, 184 (1958).
11) M. Wilimowski, J. Gieldanowski, A. Pelczarska : Arch. Immunol. Terap. Doświadczalnej, 8, 377 (1960) (C. A., **55**, 3810i (1961)).

action of chloral hydrate, hexobarbital, or phenobarbital. These reports are very interesting in regard with the present experiments. It is progressing to investigate the relationship between the chemical structure and stimulatory effect on the drug-metabolism.

The authors are indebted to Teikoku Hormone Mfg. Co. for their supply of testosterone and to Shionogi & Co. for that of cyclobarbital.

Summary

Some factors affecting on the metabolism of cyclobarbital (EHB) by the rat liver preparation were examined.

Sex difference was especially shown in the formation of 3-OH-EHB, but no difference in that of 3-keto-EHB was shown.

The pretreatment of female rats with EHB enhanced both the formation of 3-OH-EHB and that of 3-keto-EHB in approximately same proportion as controls. However, the pretreatment with 3-OH-EHB increased the formation of 3-OH-EHB and decreased that of 3-keto-EHB from EHB. The conversion of 3-OH-EHB to 3-keto-EHB was depressed by the pretreatment with testosterone or 3-OH-EHB. On the other hand, the conversion of 3-keto-EHB to 3-OH-EHB was stimulated by such a treatment. Another metabolite of EHB, 3-keto-EHB, has not stimulatory effect on the EHB-metabolism.

The removal of the adrenal glands did not prevent the stimulatory effect of EHB.

(Received July 14, 1961)

UDC 547.852.2.07

153. **Shigeru Sako** : Syntheses of Pyridazine Derivatives. I. The Reactivity of Chlorine Atoms in 3- and 6-Positions of 3,6-Dichloropyridazine 1-Oxide.

(*National Institute of Hygienic Sciences*[*1])

In the previous paper,[1] it was shown that 3,6-dichloropyridazine (I) and 3-alkoxy-6-chloropyridazines gave their 1-oxide derivatives by N-oxidation. In this paper, reactivity of chlorines of 3,6-dichloropyridazine 1-oxide (II) in nucleophilic substitution will be described.

When (II) was reacted with one equivalent of sodium ethoxide at room temperature, two isomers, (A) and (B), were obtained. The former melted at 115~116°, yield 72%, and the latter melted at 138~139°, yield 11%. (A) was identified by mixed melting point determination and by comparing its infrared spectrum with 3-ethoxy-6-chloropyridazine 1-oxide (IIIb), prepared in the previous work.[1] Accordingly, a structure of 6-ethoxy-3-chloropyridazine 1-oxide (IVb) was assigned to (B). This fact seemed to be rather curious, because these seemed to have little contribution of −M effect of N-oxide, to the reactivity of 6-chlorine atom, and only −M effect of tertiary nitrogen to 3-chlorine atom could be seen. But anyhow, the chlorine atom in 3-position was found more

*1 Tamagawa-Yoga-machi, Setagaya-ku, Tokyo (佐子 茂).

1) T. Itai, S. Sako : This Bulletin, **10**, 989 (1962).