4-Methyl-6(5*H*)-phenanthridone (XVI)—A mixture of 0.37 g. of (XV) and 0.2 g. of 10% Pd-C was heated in a metal bath at 300~310° for 15 hrs., cooled, and the content was dissolved in $CHCl_3$, removing Pd-C. The $CHCl_3$ solution was washed consecutively with 5% NaOH, 5% HCl, and water, dried over anhyd. Na_2SO_4, and $CHCl_3$ evaporated. The residue (0.16 g.) was recrystallized several times from $CHCl_3$ and gave 0.05 g. of (XVI), m.p. 240.5~242°, undepressed on admixture with an authentic sample, m.p. 240~242°. *Anal.* Calcd. for $C_{14}H_{11}ON$: C, 80.4; H, 5.3; N, 6.7. Found: C, 80.43; H, 5.36; N, 6.60.

4,10b-Dimethyl-4-hydroxymethyl-1,2,3,4,4a,5,6,10b-octahydrophenanthridine (XVII)—The lactam (XV) was treated with ether solution of CH_2N_2 to prepare the methyl ester (not crystallized) and the solution of 0.46 g. of this methyl ester dissolved in 40 cc. of dehyd. dioxane was boiled with 0.46 g. of $LiAlH_4$ for 8 hrs. Ice water was added to this mixture cautiously, inorganic matter was filtered off, and the filtrate was evaporated under a reduced pressure. The residue was recrystallized from acetone-petr. ether mixture to (XVII), m.p. 128.5~129°. *Anal.* Calcd. for $C_{16}H_{23}ON$: C, 78.3; H, 9.5; N, 5.7. Found: C, 78.74; H, 9.35; N, 5.64. $[\alpha]_D^{15}$ −44.5°(c=1.664, EtOH). U.V. λ_{max} mμ (log ε): 267 (2.58), 274 (2.51).

Hydrochloride: m.p. 215°(from dil. EtOH).

Diacetate: m.p. 100~101°(from ether-petr. ether). *Anal.* Calcd. for $C_{20}H_{27}O_3N$: C, 72.9; H, 8.3; N, 4.3. Found: C, 73.18; H, 8.13; N, 4.19.

Summary

Oxidation of methyl 9,10-dioxodeisopropylallodehydroabietate (II) with alkaline hydrogen peroxide yielded a dicarboxylic acid (III). Thermal decomposition of the tricarboxylic acid (IV) obtained by saponification of (III) afforded two kinds of fluorenone derivatives, (VII), m.p. 147~149°, and (VIII), m.p. 130~131°. Structures of these compounds were examined, together with the Beckmann rearrangement of the oxime of (VIII).

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47. Eigo Takabatake: Metabolism of Drugs. X.*[1] The Relationship between Hypnotic Activity and Metabolism of Ethylhexabital. (1). The Influence of Species, Sex, and Age on the Activity of Liver to Metabolize Ethylhexabital and the Isolation of an *in vitro* Metabolite.

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It is known that the duration of barbiturate hypnosis is regulated by the rate of their biotransformation in the body and influenced by several conditions. It was previously shown[1] by the use of paper chromatography and ultraviolet spectrophotometry that ethylhexabital J.P. (5-(1-cyclohexenyl)-5-ethylbarbituric acid, EHB) was converted by rat liver slices *in vitro* to 3-keto-EHB which was pharmacologically inactive. The isolation and identification of an *in vitro* metabolite as 3-keto-EHB are described in the present paper. The relationship between the duration of EHB hypnosis and the EHB-metabolizing activity of liver slices is also shown with regard to species, sex, and age differences.

Materials and Methods

Animal: A litter mates of rats born in the Institute were used. Other animals were used after breeding for at least one week under our controlled conditions. All animals were fasted for

* This constitutes a part of a series entitled "Metabolism of Drugs" by H. Tsukamoto.

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1) Part IX. E. Takabatake: J. Pharm. Soc. Japan, **76**, 511(1956).

about 15 hrs. before the experiment.

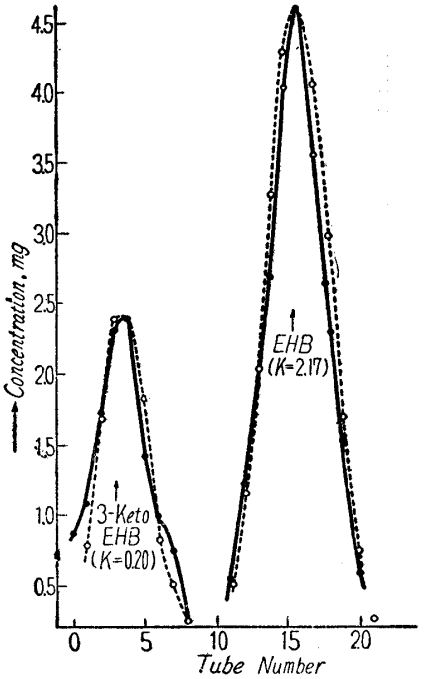
EHB was intraperitoneally injected immediately after preparing aqueous solution containing 1.1 equivalents of NaOH. The duration of hypnosis was determined as the period during which the animals would lie without resistance on their back or sides.

The *in vitro* experiments were performed according to the methods described in the preceding report[1] (liver slices : 500 mg., EHB added : 500 γ, Krebs-Ringer phosphate buffer containing 0.2% of glucose, pH 7.4, total volume 10 cc., 38°, 3 hrs., in oxygen atmosphere).

Results

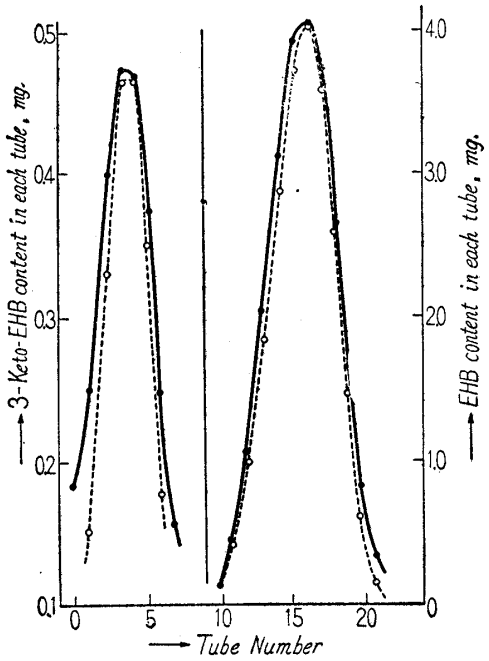
I. Isolation and Identification of an *in vitro* Metabolite

Male rabbits weighing about 2.5 kg. were sacrificed by injection of air into the marginal ear vein. The liver was immediately removed, minced, and passed through a sieve (No. 3, J. P.). Then, the liver preparation was suspended in 300 or 400 cc. of Krebs-Ringer phosphate buffer (pH 7.4) containing 0.2% of glucose. EHB was dissolved in *N* NaOH, diluted with buffer to 100 cc., and added to the liver suspension. After incubation for 3 hrs. at 38° with gentle stirring and passing of oxygen, the incubation flask was immersed in a boiling water bath for 15 mins. After the flask was cooled and the content filtered, the filtrate was acidified with conc. HCl and continuously extracted with ether for 15 hrs. Ether extract was washed with acidic water, dried over Na_2SO_4, and evaporated. The residue was subjected to 23 transfers in counter-current distribution, the solvents being an equal volume (10 cc.) of BuOH and borate buffer of pH 9. After distribution, the quantity of barbiturate in each tube was determined as follows : The upper layer was extracted with 0.1*N* NaOH and adjusted with 0.2*M* borate to pH 11, the lower layer was adjusted with 0.1*N* NaOH to pH 11, and their ultraviolet absorptions were measured at 230, 239, and 250 mμ. From the optical density at 239 mμ, the quantity of barbiturate was determined. In the distribution pattern of extract from *in vitro* metabolism experiment, there were two peaks indicating the presence of 3-keto-EHB (max. at tube 4) and EHB (max. at tube 16) (see Fig. 1).



—o— Experimental

Fig. 1. Counter-current Distribution of the Ether Extract from Incubation Mixture of EHB and Minced Rabbit Liver (23 Transfers)



------o------ Theoretical

Fig. 2. Counter-current Distribution of the Ether Extract from Incubation Mixture of EHB and Minced Rat Liver (23 Transfers)

For isolation of 3-keto-EHB the contents of tubes 2 to 9 were pooled. After BuOH in the upper layer was evaporated under a reduced pressure, the residue was combined with lower aqueous phase, acidified with conc. HCl and continuously extracted with ether. The ether was evaporated and the residual material was recrystallized from MeOH to yield colorless plates which melted at 222° (decomp.) A mixed melting point of the isolated product with the sample obtained from rabbit

urine showed no depression. Unchanged EHB was also isolated from the pooled content of tubes 13 to 20 by the same procedure as for 3-keto-EHB. In the experiment with rat liver, both EHB and 3-keto-EHB were identified (Fig. 2). Table I summarizes the results.

Table I. Metabolism of EHB by Rabbit Liver

Expt. No.	I	II	III
Minced liver (g.)	64	86	110
EHB added (mg.)	100	100	200
Volume of incubation medium (cc.)	400	400	500
EHB obtained as crystals (mg.)	37	43	47
3-Keto-EHB obtained as crystals (mg.)	14	39	23

II. Species Differences

Adult rats, mice, rabbits, and guinea pigs of both sexes were examined. The duration of hypnosis produced by injection of EHB in a dosage of 80 mg./kg. of body weight was the shortest in male rat and the longest in guinea pigs of both sexes. The duration in other species was nearly equal. In regard to the EHB-metabolizing activity, which was estimated from the amounts of EHB that remained and 3-keto-EHB (pharmacologically inactive) formed in the *in vitro* experiment with liver slices, the male rat liver was the strongest and the guinea pig liver was the weakest. The data are presented in Table II. It was found that the activity of liver slices to metabolize EHB was inversely related to the duration of EHB hypnosis. Thus, the species difference in the duration of EHB hypnosis could be explained in the terms of EHB-metabolizing activities of their liver. The marked sex difference was found only in the rat, where the male rat metabolized more rapidly than the female.

Table II. Duration of EHB Hypnosis and EHB-Metabolizing Activity of Liver Slices in Various Animal Species

Species	Sex	No. of animals	Body wt. (g.)	Duration (min.)*	No. of animals	Body wt. (g.)	3-Keto-EHB formed (γ)	EHB remained (γ)
Rat	Male	6	172	72	2	167	242	199
	Female	4	139	159	2	163	145	230
Mouse	Male	8	13	139	8	16	152	205
	Female	7	14	145	8	16	140	226
Rabbit	Male	4	1975	147	3	2480	133	205
	Female	4	3180	121	2	2980	143	—
Guinea pig	Male	9	340	173	2	307	108	228
	Eemale	5	322	168	2	320	129	215

*EHB was intraperitoneally injected in a dosage of 80 mg./kg.

III. Influence of Sex and Age of Rats on the Metabolism of EHB

The results are shown in Table III. The duration of hypnosis produced by intraperitoneal injection of EHB in a dosage of 100 mg./kg. of body weight was repeatedly measured in a litter A consisting of five males and four females with about 2-week intervals over 6 months. Two measurements, (a) and (b), with one-day interval were performed in each experiment. The percentage of duration in each (a) determination to the duration of 25-days (experiment No. Ia) was shown as G-a ratio. In G-b ratio, the duration at 27-day-olds (experiment No. Ib) was adopted as the standard. The G-ratio based on the growth gradually decreased in both sexes as the rats grew, but the decreasing rate of G-ratio in the male was more rapid than that in the female. After 75 days, the G-ratio of the male was nearly equal but that of female rapidly increased. Thus, the S-ratio, the percentage of duration of female to that of male at the corresponding age, gradually increased and reached about 300% in the 180-day-olds and the sex difference in duration of hypnosis became significant after 73 days. The duration of hypnosis considerably varied among younger rats but the variation within a litter was small. In another experiment with three litters (B, C, and D of Table III), the duration in the 25-day-olds was shorter than that of litter A but no sex difference was observed. In adult rats (E of Table III), whose ages were not known but assumed to be more than 150 days from their body weight, a marked sex difference was apparent.

The EHB-metabolizing activity of liver slices was assayed at various ages of several litter mates and the results are summarized in Table IV. The activity gradually increased in both sexes as rats grew, but the increasing rate in the male was more rapid than that in the female. The sex difference in metabolizing activities of liver slices became significant after 75 days. Fig. 3 shows the G-ratio curves of hypnotic duration and of metabolizing activity.

IV. The Effect of Preadministration on the Metabolism of EHB

As shown in Table III, the duration of hypnosis in each (b) determination was shorter than that

Table III. Influence of Sex and Age of Rats on the Duration of EHB Hypnosis

Litter	Expt. No.	Date 1956	Age (days)	Male: Body wt. (g.)	Male: Duration** (min.)	Male: G-a ratio (%)	Male: G-b ratio (%)	Male: R (%)	Female: Body wt. (g.)	Female: Duration** (min.)	Female: G-a ratio (%)	Female: G-b ratio (%)	Female: R (%)	S ratio (%)	Significance of sex difference (p)
A (♂10 ♀10)***	I a	17/V	25	23	214	100			24	196	100			92	>.05
	I b	19/V	27	24	143		100	67	25	141		100	72	99	〃
	II a	2/VI	41	43	123	57.5			43	140	71.4			115	〃
	II b	4/VI	43	44	85		59.4	69	44	95		67.3	67	112	〃
	III a	18/VI	57	76	99	46.2			72	120	61.2			121	〃
	III b	20/VI	59	78	72		50.3	73	74	89		63.1	74	123	〃
	IV a	4/VII	73	116	78	36.4			105	119	60.7			152	.05~.01
	IV b	6/VII	75	115	44		30.8	56	105	89		63.1	75	202	<.01
	V a	19/VII	88	139	72	33.6			125	161	82.1			224	<.001
	V b	21/VII	90	141	55		38.4	76	127	103		73.1	64	188	〃
	VI a	19/IX	150	168	63	29.4			151	147	75.0			234	〃
	VI b	21/IX	152	172	56		39.2	89	156	125		88.7	85	223	〃
	VII a	4/X	165	208	78	36.4			173	260	122.0			334	〃
	VII b	6/X	167	207	71		49.7	91	170	210		149.0	81	296	〃
	VIII a	18/X	179	220	93	43.4			174	323	165.0			348	〃
	VIII b	20/X	181	214	62		43.4	67	172	203		144.0	63	328	〃
B	(♂2	♀4)	26	35	89				37	84				94	>.05
C	(♂2	♀1)	26	38	125				41	112				90	〃
D	(♂4	♀3)	26	30	127				28	123				97	〃
E*	(♂10	♀16)	150	189	68				126	223				328	<.01

* The rats of this group were not litter mates. Their accurate ages were not known but assumed to be more than 150 days.

** EHB was intraperitoneally injected in a dosage of 100 mg./kg. of body weight.

*** Number of rats is given in parentheses.

Table IV. Influence of Sex and Age of Rats on the EHB-Metabolizing Activity of Liver Slices.

Age (day)	Male: No. of rats	Male: Body wt. (g.)	Male: EHB remained (γ)	Male: 3-Keto-EHB formed (γ)	Female: No. of rats	Female: Body wt. (g.)	Female: EHB remained (γ)	Female: 3-Keto-EHB formed (γ)
26	8	29	245	177	4	25	209	163
37	2	36	242	174	4	32	267	165
60	9	74	211	208	6	63	223	181
75	7	69	147	259	8	59	208	198
Adult*	20	174	189	231	15	137	314	142

* The ages of rats of this group were not accurately known.

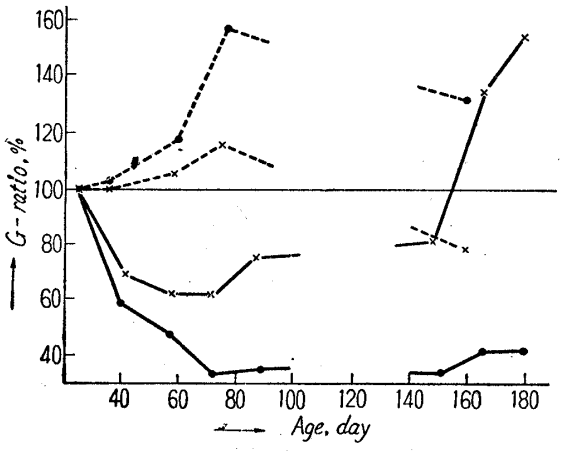


Fig. 3.

Effect of Age of Rats on the Duration of EHB Hypnosis and the EHB-Metabolizing Activity of Liver Slices

——— Duration

------ Metabolizing activity

• Male × Female

in corresponding (a). The R-ratio, the percentage of duration in (b) to that in (a), was about 70%. The results presented in Table V indicate that the metabolizing activity of liver of rats which had once received EHB two days previously was significantly higher than that of the control of both sexes. Thus, it is concluded that the shortening in duration by preadministration of EHB would be mainly due to the increase in the metabolizing activity of the liver.

Table V. The Effect of Repeated Administration of EHB on the EHB-Metabolizing Activity of Liver Slices

	Male				Female			
	No. of rats	Body wt. (g.)	EHB remained (γ)	3-Keto-EHB formed (γ)	No. of rats	Body wt. (g.)	EHB remained (γ)	3-Keto-EHB formed (γ)
Control	4	105	102	216	4	78	247	116
EHB preadministration	4	108	88	246	4	77	199	181
Significance of difference*			NS	S			S	HS

* NS : not significant, HS : highly significant ($p<.01$), S : significant ($p<.05$).

Discussion

It was ascertained by the isolation and identification of a metabolite that EHB was converted to 3-keto-EHB *in vitro* as *in vivo*.[2~4] Some *in vitro* metabolites of barbiturates were isolated and identified with *in vivo* metabolites, e.g., keto-Evipal from Evipal[5] or pentobarbital from thiopental.[6]

The duration of hypnosis produced by barbital which was not metabolized in the body was nearly the same in various animal species. On the other hand, the species difference was found in the duration of hypnosis produced by Evipal or pentobarbital which was rapidly metabolized. Brodie, *et al.*[7] found that there was an inverse relationship between the activity of Evipal-oxidizing enzyme system in microsomes of the liver and the duration of drug action and that Evipal was metabolized extremely rapidly in the mouse and slowly in the dog. In regard to EHB, the inverse relationship between the duration of hypnosis and the metabolizing activity of liver slices was evident as shown in this experiment.

The metabolism of EHB was inhibited by β-diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525-A)[8] the same as those of a number of drugs including Evipal.[7] In spite of the similarity between EHB and Evipal in chemical structure and the behavior to SKF 525-A, the animal species which metabolized EHB most rapidly was not the mouse but the male rat. The cause of this difference is unknown, but it is of interest to consider that EHB was mainly biotransformed to 3-keto-EHB and that, on the other hand, a principal metabolite of Evipal was 3-hydroxy-Evipal.[9]

In both deamination of amphetamine[10] and demethylation of ephedrine,[11] Axelrod found that the rabbit liver microsome had the strongest activity and that the heat-labile inhibiting factor was present in dog, guinea pig, or rat liver mitochondria. In the case of N-demethylation of narcotic drugs,[12] *l*-methadone and meperidine were most rapidly

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metabolized by rabbit liver and morphine, the most by rat liver. All these metabolic pathways require both reduced triphosphopyridine nucleotide (TPNH) and oxygen and are, in general, inhibited by SKF 525-A.[7] However, the animal species indicating the strongest activity for metabolism was variable according to the chemical structure of compounds. It was shown that the different animal species metabolized some drugs in entirely different pathways.[7] The metabolic pathways of EHB to other than 3-keto-EHB are unknown, but no other metabolites have been identified, at least in rats and rabbits.

Sex difference in the duration of EHB hypnosis and in the metabolizing activity of EHB were found only in the adult rat and not in immature rat, adult mouse, rabbit, or guinea pig. In the cases of Amytal, pentobarbital, and Evipal hypnosis, sex difference was found only in the rat but not in dog, cat, rabbit, guinea pig, mouse, or frog.[12,13] Komiya and Fukushima[14] reported that no sex difference was observed in the mouse receiving thiopental. It was said that the sex difference in pentobarbital hypnosis was markedly apparent in the adult rat weighing 200~500 g.[15,16] According to Moir[17] the resistance to pentobarbital anesthesia in the very young male rat was lower than that in the female of the corresponding age but then it was intensified with growth, probably under the influence of male sex hormone. The effect of sex hormones on the metabolism of EHB in the liver will be discussed in the next paper of this series.

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Summary

The isolation of an *in vitro* metabolite of ethylhexabital (EHB) and its identification as 3-keto-EHB were described.

It was found that there was an inverse relationship between the duration of EHB hypnosis and the EHB-metabolizing activity of liver slices. Of the animal species used in this study, the adult male rat slept for the shortest period and most rapidly metabolized EHB and *vice versa* for the guinea pig. The sex difference in the duration of EHB hypnosis and EHB-metabolizing activity of liver slices were found only in the adult rat. The liver of male rats more rapidly metabolized EHB than that of female rats.

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