46. Hisao Tsukamoto, Eigo Takabatake, and Hidetoshi Yoshimura : Metabolism of Drugs. I. The Metabolic Fate of Ethylhexabital (5-Cyclohexenyl-5-ethylbarbituric Acid). (1).

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Studies on the metabolism of drugs are essential for clarifying the detoxication mechanism of an organism and for forensic analyses. The present investigation was undertaken in order to isolate and identify the metabolic products of drugs and to determine quantitatively the rate of detoxication in organisms. The barbiturates were chosen for this study because they were widely used as hypnotics and frequently became the object of discussion in forensic chemistry. The first drug studied was Ethylhexabital J. P. (EHB, 5-cyclohexenyl-5-ethylbarbituric acid, Phanodorm) which was difficult to detect in forensic chemistry.

A great many investigations on the metabolic fate of barbiturates were reviewed by Maynert and van Dyke[1]. Fretwurst, Halberkann, and Reiche[2] found that 2~7% of EHB was eliminated unchanged and that 12~19% was excreted as a non-toxic compound which was believed to be 5-cyclohexenonyl-5-ethylbarbituric acid on the basis of chemical analysis, but no detailed description was given as to its chemical structure.

It is shown in this paper that a metabolite of EHB obtained from the urine of rabbits receiving EHB is identical with an oxidation product of EHB with chromic acid.

Methods and Results

Isolation of EHB-metabolite (EHB-M) from the Urine of Rabbits—Calcium salt of EHB (Adorm, Shionogi) in the doses of 70~200 mg./kg. body weight was administered by stomach tube to rabbits weighing 2~2.5 kg. after fasting for 18~24 hours. The animals were fed *ad. lib.* 3 hours after the administration and their urine was collected for 24 hrs. into a bottle containing a few drops of toluene. In these doses no hypnotic action was shown. The collected urine (pH 8.0 or so) was filtered through the cotton, brought to pH 5.0 with conc. H_2SO_4, and extracted continuously with ether for 20 hours. In other cases the acidified urine was shaken with isoamyl alcohol or ethyl acetate, centrifuged, and the organic solvent layer siphoned off. A reddish oily substance left after evaporating the solvent was recrystallized from methanol to colorless plates, m.p. 222°(decomp.), EHB-M. *Anal.* Calcd. for $C_{12}H_{14}O_4N_2$: C, 57.60; H, 5.60; N, 11.20. Found : C, 57.83; H, 5.48; N, 11.22.

The reddish-brown oily substance left after removal of the crystals was dissolved in acetone and chromatographed through an alumina column and a small quantity of EHB-M was recovered from the methanol eluate. Unchanged EHB, however, could not be isolated. The yield of EHB-M from the urine is shown in Table I.

Table I. Isolation of Ethylhexabital-Metabolite (EHB-M) from the Urine of Rabbits Receiving Ethylhexabital (EHB).

No. of rabbits	Av. body weight (g.)	Dose Individual (mg./kg.)	Dose Total (mg.)	Volume of urine (cc.)	Solvent for extraction	Yield of metabolite Weight (g.)	Yield of metabolite Ratio to EHB (%)
3	2290	70	460	1450	Ether	83	18.1
3	2263	150	1018	825	Ether	105	9.3
3	2380	150	1071	770	Ether	90	9.3
3	2510	150	1130	350	*iso*-AmOH	142	12.6
4	2960	200	1780	—	AcOEt	310	17.4

Oxidation of EHB with Chromic Acid—To a well stirred solution of EHB (20 g.) in hot glacial AcOH (200 cc.) was added dropwise a solution of anhyd. CrO_3 (17 g.) in glacial AcOH (130 cc.) and

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1) E. W. Maynert, H. B. van Dyke : Pharmacol. Rev., **1**, 217 (1949).
2) F. Fretwurst, J. Halberkann, F. Reiche : Münch. med. Wochschr., **79**, 1429 (1932).

water (4 cc.) during 1 hour and kept at approx. 30°. After cooling to room temperature under stirring, the reaction mixture was allowed to stand overnight, and the green solution was poured into 1.5 L. of ether. The ether layer was separated and washed with water till it became colorless. After evaporating ether, remaining AcOH was removed under a reduced pressure, and to the residue was added 400 cc. of water. The crystalline product that deposited from the solution on cooling and obtained by concentration under a reduced pressure, was recrystallized from MeOH. Four g. of colorless plates, m.p. 221～222°(decomp.), was obtained. The aqueous washing was extracted continuously with ether for 15 hours after removal of AcOH *in vacuo* and addition of a small amount of water. The crystals obtained by evaporation of ether were recrystallized from MeOH to colorless plates, m.p. 221～222°(decomp.). Total yield, 5.3 g. or 25%. The melting point of this product was not depressed by admixture with a sample, EHB-M, obtained from the rabbit urine, and the ultraviolet and infrared spectra of these two substances were identical (Figs. 1 and 2).

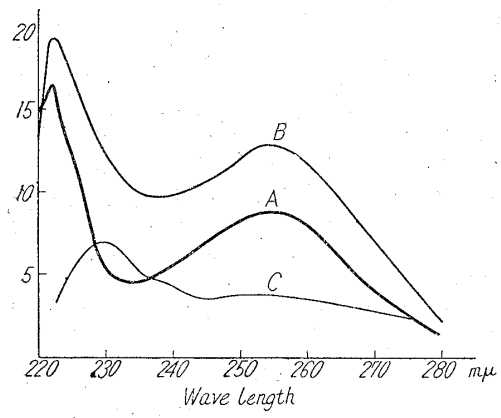
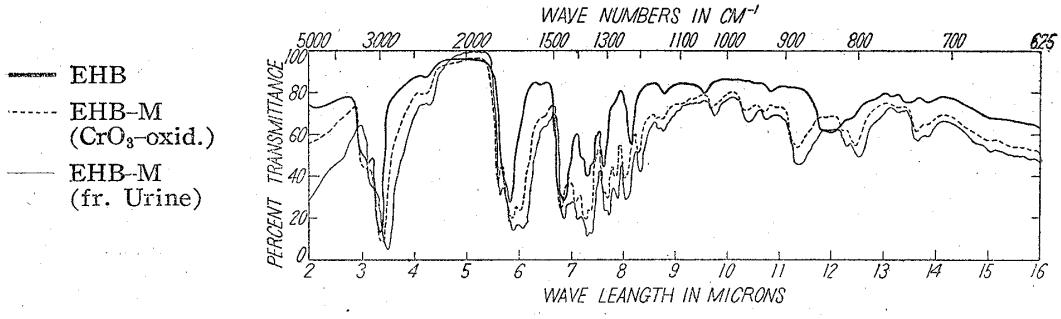


Fig. 1. Ultraviolet Absorption Spectra of Ethylhexabital (EHB) and its Metabolite (EHB-M)

Curve A indicates the spectrum of EHB, Curve B, EHB-M, and Curve C, the difference of absorption (absorption of EHB-M minus that of EHB).

Fig.2. Infrared Absorption Spectra



Properties of EHB-M—EHB-M is soluble in MeOH, EtOH, BuOH, *iso*-AmOH, and acetone, slightly soluble in ether, AcOEt, and $CHCl_3$, and practically insoluble in CCl_4 and ethylene dichloride.

The color reaction with Cu-pyridine reagent (a mixture of 4 cc. of 10% $CuSO_4$, 1 cc. of pyridine, and 5 cc. of water) or Cu-quinoline reagent (a mixture of 4 cc. of 10% $CuSO_4$ and 6 cc. of water saturated with quinoline) which is characteristic of barbiturates was positive in both EHB and EHB-M. As shown in Fig. 1 the ultraviolet spectra of both dissolved in 0.5*N* NaOH solution exhibited a typical peak of barbiturates at 255 mμ. The difference of absorption between the two substances was the greatest at 230 mμ. Their infrared spectra are shown in Fig. 2.

Derivatives of EHB-M—2,4-Dinitrophenylhydrazone : To a solution of EHB-M in MeOH was added a solution of 2,4-dinitrophenylhydrazine-HCl in MeOH and the mixture was warmed on a water bath for 5 minutes. Reddish-orange crystals deposited after cooling were recrystallized from AcOEt to m.p. 275°(decomp.). *Anal.* Calcd. for $C_{18}H_{18}O_7N_6$: C, 50.26; H, 4.18; N, 19.53. Found : C, 50.32; H, 4.40; N, 19.97. Recrystallization from glacial AcOH yielded an adduct with 1 mole of AcOH. *Anal.* Calcd. for $C_{18}H_{18}O_7N_6 \cdot C_2H_4O_2$: C, 48.98; H, 4.49; N, 17.14. Found : C, 48.96; H, 4.54; N, 16.92.

Acetylation : An attempt to acetylate EHB-M with Ac_2O was unsuccessful and all starting material was recovered.

Catalytic reduction : EHB-M (0.15 g., 0.0006 mole) dissolved in EtOH (20 cc.) was catalytically reduced using 0.05 g. of 2% Pd-C, absorbing 14.2 cc. of H_2 (0.0006 mole at 15°). After evaporating EtOH, the residue was recrystallized from water to yield small needles of m.p. 212° (2H-EHB-M). *Anal.* Calcd. for $C_{12}H_{16}O_4N_2$: C, 57.24; H, 6.35; N, 11.11. Found : C, 57.26; H, 6.36; N, 11.04.

2,4-Dinitrophenylhydrazone of 2H-EHB-M was obtained by the same procedure as for EHB-M and recrystallized from AcOEt to m.p. 265°(decomp.). *Anal.* Calcd. for $C_{18}H_{20}O_7N_6$: C, 50.00; H,

4.63; N, 19.44. Found: C, 50.28; H, 4.35; N, 19.33.

Paper Chromatography of EHB and EHB-M—Several barbiturates including EHB and EHB-M were developed with BuOH saturated with 5*N* NH_4OH on a filter paper (Toyo Roshi No. 50, 1×40 cm.) by the ascending method. The dried sheets were sprayed with Cu-pyridine or -quinoline reagent and the zones containing barbiturates appeared purple against a lighter blue background. In Table II the Rf values of several barbiturates are shown. The barbiturates examined were located by this method in amount as low as 100 γ.

Table II. Rf Values of Several Barbiturates

Barbiturate	Rf
Barbital	0.49
Phenobarbital	0.54
Allobarbital	0.59
Methylhexabital	0.74
Ethylhexabital (EHB)	0.65
Ethylhexabital-Metabolite (EHB-M)	0.24

Biological Activity of EHB-M—The drugs were dissolved in redistilled water with 1.1 equivalents of NaOH immediately before use and administered intraperitoneally to mice weighing 15~20 g. Death within 24 hours after the administration was ascribed to the toxicity of the drugs. EHB administered in a dose of 0.1 mg./g. produced in 10 minutes a typical hypnotic action lasting about 2 hours, but EHB-M, even when administered in a larger dose of 5.0 mg./g., caused no apparently noticeable action. Median lethal dose (LD_{50}) of EHB-M estimated by the method of Finney[3] was 2.59 mg./g. with fiducial limits of 2.23 and 3.03 mg./g. at P=0.05. From the calculation of the relative potency, it was estimated that 1 mg. of EHB-M was equivalent to 0.045 mg. of EHB. In other words EHB-M was about 1/22 as toxic as EHB.

Table III. Lethal Dose of EHB-M

Dose, mg./g.	No. of mice	No. of dead mice
5.0×0.8^6	8	0
5.0×0.8^5	8	2
5.0×0.8^4	8	1
5.0×0.8^3	8	3
5.0×0.8^2	8	6
5.0×0.8	8	7
5.0	8	8

LD_{50}=2.59 mg./g. Fiducial limit: 2.23~3.03 mg./g.

Table IV. Relative Potency of EHB-M to EHB

	Dose, mg./g.	No. of mice	No. of dead mice
Ethylhexabital (EHB)	0.135×0.9	12	3
	0.135	12	6
Ethylhexabital-metabolite (EHB-M)	3.15×0.9	12	4
	3.15	12	8

Discussion

Unchanged EHB was detected by Fretwurst, *et al.*[2] in a 12-day urine of humans receiving total amount of 4~5 g. of EHB for 10 days, but undetectable in the rabbit urine under the present experimental conditions. It is known that there is some correlation between the *in vivo* stability of the individual barbiturates and their duration of action. On this basis a large majority of long-acting drugs such as barbital are eliminated unchanged and drugs of the group with moderate or short duration of effect, such as EHB, Pentobarbital, Evipal, and so on, on the contrary, are more unstable and appear in the urine only in very small amounts. The metabolic process may be varied in different species, so that the difference between Fretwurst's results and ours need not be considered as a matter of serious consequence, though of course the problem is expected to be settled by future research.

The metabolite of EHB obtained by Fretwurst, *et al.* and that by us well agree in

3) D. J. Finney: J. Pharmacol. Exptl. Therap., **104**, 440 (1952).

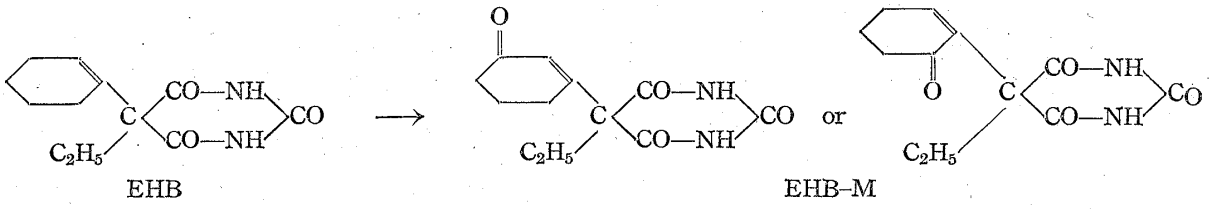
the yield, m.p., and analyitcal values, and is believed to be 5-cyclohexenonyl-5-ethylbarbituric acid which is formed by the introduction of one carbonyl group into the cyclohexene ring of EHB.

It is said that Medomin[4] (5-cycloheptenyl-5-ethylbarbituric acid) which resembles EHB in structure is metabolized to 5-cycloheptenonyl-5-ethylbarbituric acid and that Evipal[5] (5-cyclohexenyl-1,5-dimethylbarbituric acid) is demethylated and/or a carbonyl group introduced into the molecule. However, no one has defined the position of the carbonyl group.

From the results of the color reaction with Cu-pyridine or -quinoline reagent and ultraviolet absorption spectrum in 0.5 *N* NaOH solution, it is presumed that EHB-M retains the barbituric acid structure. The ability to absorb an equivalent mole of hydrogen on catalytic reduction shows that the double bond in cyclohexene ring remains unchanged in EHB-M. The facts that both EHB-M and 2H-EHB-M form 2,4-dinitrophenylhydrazone and are not acetylated shows that one carbonyl group is introduced into EHB. As shown by Bush, Butler, and Dickison[5] in Evipal and its metabolites, in the case of EHB and EHB-M, the augmented absorption due to the carbonyl group (absorption of EHB-M minus absorption of EHB) is maximal at about 230 mμ. This absorption behavior suggests that EHB-M may be α,β-unsaturated ketone.

A metabolite of Amytal (5-isoamyl-5-ethylbarbituric acid) was isolated by Maynert[6] from the urine of dogs, synthesized by the oxidation with chromic acid, and identified as 5-(3-hydroxyisoamyl)-5-ethylbarbituric acid. The oxidation of EHB with chromic acid also gives a product which is identical with a metabolite obtained from the urine of rabbits. It is apparent that both biological metabolite and synthetic product are identical because there is no depression in the melting point on admixture and because these two substances give identical pattern in the ultraviolet and infrared spectra.

As shown by Treibs and Schmidt[7], and by Mousserson, Jacquier, and Winternitz[8], when the cyclohexene derivatives are treated with chromic acid the methylene group neighboring the double bond is generally oxidized to a ketone or alcohol. Thus, it is assumable that the position of the ketone in EHB-M is at 3 or 6 of the cyclohexene ring as shown in Fig. 3.



EHB EHB-M

Fig. 3.

For the identification of EHB-M and EHB, paper chromatography was tried by the method of Algeri and Walker[9]. The Rf values of several barbiturates developed by butanol-5*N* NH_4OH solvent system are too close to be separated from each other, but the Rf value of EHB-M is very remote from EHB. Thus, this method is very useful in identification.

Pulver[4] found that 1% of Medomin administered was excreted in the urine as a metabolite which is 1/20 as toxic to mice as Medomin and has no hypnotic action, and similarly, EHB-M is 1/22 as toxic as EHB and has no hypnotic action. These results

4) R. Pulver : Schweiz. med. Wochschr., **73**, 124 (1943).
5) M. T. Bush, T. C. Butler, H. L. Dickison : J. Pharmacol. Exptl. Therap., **108**, 104 (1953).
6) E. W. Maynert : J. Biol. Chem., **195**, 397 (1952).
7) W. Treibs, H. Schmidt : Ber., **61**, 459 (1928).
8) Mousserson, Jacquier, Winternitz : Compt. rend., **224**, 1230 (1947); C. A. **41**, 6536 (1947).
9) E. J. Algeri, J. T. Walker : Am. J. Path., **22**, 37 (1952).

agree with that of Fretwurst[2].

The quantitative investigation on the metabolic process of EHB is in progress.

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Summary

A metabolite of ethylhexabital isolated from the urine of rabbits receiving ethylhexabital was identified with an oxidation product of ethylhexabital with chromic acid. This metabolite is about 1/22 as toxic as ethylhexabital and has no hypnotic action. The chemical structure of EHB-M was disscussed and assumed as 5-cyclohexenonyl-5-ethylbarbituric acid.

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47. **Nobuo Ikekawa, Yoshihiro Sato, and Taizo Maeda**: Studies on the Coal Tar Bases. VII[1]. Basicity of Methylpyridines.

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It is well established that the methyl group in the α- or γ-position of the pyridine ring is more reactive than that in the β-position because of the influence of the nitrogen forming the pyridine nucleus. On the other hand, it is interesting that the basicity of methylpyridine is changed by a change in the position and number of the methyl group on the pyridine ring, namely, the effect of the methyl group on the basicity of the nitrogen of pyridine ring. In the previous papers of this series[2], synthesis of polymethylpyridines was described and some of the pyridines were isolated from coal tar bases of low-temperature coke[3]. The present investigation was undertaken to determine the dissociation constants of all methylpyridine isomers and to compare the basicity of these isomers.

Although the literature contains several references to the determination of the dissociation constants of pyridine and picoline, the values mentioned are varied. Brown and his coworkers studied the basicity of pyridine, three picolines, and 2,6-lutidine toward trifluoroboride[4] and trimethyl boride[5]. Herington[6] determined the dissociation constants of these bases from absorption spectra. Recently, Gero and Markham[7] compared the pK_a values of pyridine and five methylpyridines obtained from pH titration curves in aqueous solution and concluded that the pK_a value of methylpyridine was obtainable from the following experimental equation

$$pK_a = pK_{py} + 0.82\,\gamma + 0.73\,\alpha - 0.03\alpha(\alpha - 1)$$

in which pK_{py} represents the pK_a of pyridine (5.23) and γ or α is the number of methyl group in γ- or α-position, respectively. A methyl group manifests its electron-donating character by increasing the electron density at the N atom (expressed in the pK_a) by a

* Hakata, Fukuoka (池川信夫, 佐藤良博, 前田泰三).

1) Part VI: This Bulletin, **1**, 283(1953).
2) K. Tsuda, *et al.*: *Ibid.*, **1**, 122, 126, 142, 283(1953).
3) N. Ikekawa: *Ibid.*, **1**, 149(1953).
4) H. C. Brown, H. I. Schlesinger, S. Z. Cardon: J. Am. Chem. Soc., **64**, 325(1942).
5) H. C. Brown, G. K. Barbars: *Ibid.*, **69**, 1137(1947).
6) E. F. G. Herington: Discussions Faraday Soc., **9**, 26(1950).
7) A. Gero, J. J. Markham: J. Org. Chem., **16**, 1835(1951).