UDC 615.782.54-092.2

28. Yukio Kuroiwa : Studies on the Metabolic N-Demethylation. I. The Metabolic Fate of Hexobarbital by Rabbit Liver Slice.

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According to Tsukamoto, *et al.*,[1,2] hexobarbital (MHB) was biotransformed into such compounds as 3-keto-MHB (5-(3-oxo-1-cycylohexenyl)-3,5-dimethylbarbituric acid), 3-OH-MHB (5-(3-hydroxy-1-cyclohexenyl)-3,5-dimethylbarbituric acid), 3-keto-nor-MHB-(5-(3-oxo-1-cyclohexenyl)-5-methylbarbituric acid) and ureide, etc. Among these, 3-keto-nor-MHB as a demethylated compound was recovered[3] in about 4.9% of the administered dose from the urine of rabbit.

Cooper and Brodie[4] showed that MHB are oxidized by the action of enzymes in microsomes that have the requirement for both TPNH (reduced triphosphopyridine nucleotide) and oxygen.

The experiment described in this paper was conducted to obtain a complete picture of the metabolic pathway of N-substituted barbiturates. *In vivo* experiments showed that N-demethylated metabolites was appeared only in the form having an oxidized cyclohexenyl group, therefore in the animal body the oxidation of cyclohexenyl group seemed more plausible than oxidative N-demethylation.

In 1960 Tsukamoto, *et al.*[5] reported that any N-demethylated substance could not be recovered *in vitro* from N-substituted barbiturates such as MHB, 3-keto-MHB and 3-OH-MHB. They used microsomal fraction of the rabbit liver as the enzyme systems.

In this experiments, rabbit liver slice was employed and the metabolites of MHB, 3-keto-MHB, 3-OH-MHB were carefully traced by means of paper chromatography. It was obvious that N-demethylated compounds were not obtained from MHB in this system, but, on the contrary a significant amount of 3-keto-nor-MHB was detected from 3-keto-MHB and 3-OH-MHB.

On the other hand, methylbarbital, which possessed C-5 side groups stable to oxidation, was demethylated both *in vivo* and *in vitro*.

From these results it was suggested that oxidation of cyclohexenyl group would be the first reaction in the metabolism of MHB, and this was followed by oxidative N-demethylation.

Experimental

Materials——Cyclobarbital (EHB) and MHB were obtained from Shionogi & Co., Ltd. and Dainippon Pharmaceutical Co., Ltd. α-3-OH-MHB (m.p. 213~215°) was furnished by Kyushu University, Pharmaceutical Institute, Tsukamoto Laboratory. 3-keto-MHB (m.p. 160~161°) was synthesized by chromic oxidation method.[6] Methylbarbital[7] (m.p. 154°) was obtained by the methylation of barbital with Me_2SO_4.

Methods——Excretion Rate of Metabolites in the Urine——EHB, MHB and methylbarbital were given as an aqueous solution containing 1.1 equiv. of NaOH by stomach tube to male albino rabbits weighing about 2.5 kg. The dose was about 1 mmole/kg. of body weight. Urine was collected every 2 hr.

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1) H. Tsukamoto, H. Yoshimura, S. Toki : This Bulletin, **4**, 368 (1956).
2) H. Yoshimura : *Ibid.*, **5**, 561 (1957).
3) H. Tsukamoto, H. Yoshimura, S. Toki : *Ibid.*, **6**, 88 (1958).
4) J.R. Cooper, B.B. Brodie : J. Pharmacol. Exptl. Therap., **114**, 409 (1955).
5) H. Tsukamoto, S. Toki, K. Toki : This Bulletin, **8**, 561 (1960).
6) H. Tsukamoto, H. Yoshimura, S. Toki : *Ibid.*, **4**, 364 (1956).
7) T.C. Butler, M.T. Bush : J. Pharmacol. Exptl. Therap., **65**, 205 (1939).

Into a glass-stoppered tube were placed 1 ml. of urine, 0.2 ml. of N H_2SO_4 and 12.5 ml. of AcOEt. After shaking the mixture, 10 ml. of AcOEt layer was taken and evaporated to dryness. The residue was dissolved in 0.8 ml. of EtOH and 0.02 ml. of this solution was applied on a paper impregnated with borate-NaOH buffer of pH 10 by the same procedure as previously reported,[3,8] The developing solvent mixture was BuOH saturated with borate-NaOH buffer of pH 10.5 and the ascending method was employed.

Table I. The Rf Values and Absorption Maxima of Barbiturates and their Metabolites

Barbiturate	Rf value	λ_{max} in borate buffer (pH 11)
3-keto-EHB	0.20	239
3-OH-EHB	0.30	239
MHB	0.76	244
3-OH-MHB	0.45	244
3-keto-MHB	0.34	240
3-keto-nor-MHB	0.13	238
Methylbarbital	0.84	244
Barbital	0.64	239

***In vitro* Metabolism of Barbiturates**——The assay for *in vitro* metabolism of MHB or methylbarbital was modified to that described for the urine.

Male albino rabbits weighing about 2.5 kg were sacrificed by injection of air into the marginal ear vein and the livers were removed and chilled in cold 0.9% NaCl solution. Liver slices of about 0.2 mm. thick was incubated in Krebs Ringer phosphate buffer (pH 7.4) solution containing 0.2% glucose.

Stoppered Erlenmeyer flasks (50 ml.) containing 1 g. of liver slice and 500 γ of substrate in a total volume of 10 ml. of Krebs Ringer phosphate buffer solution saturated by O_2 were shaken in a metabolic shaker at 37° for 3 hr. under air. After incubation, the flask contents were made acidic with 0.5 ml. of N H_2SO_4 and were immersed in a boiling water bath for 2 min. Then 2 g. of NaCl was added to the flask, and the reaction mixture was extracted with each 25 ml. of AcOEt for three times. AcOEt layer was separated, treated with anhyd. Na_2SO_4 and activated charcoal, filtered and 60 ml. of AcOEt phase was pipetted out and evaporated to dryness. The residue was dissolved in 0.8 ml. of EtOH and 0.2~0.4 ml. of the solution was chromatographed on the paper as described above. From the paper strips of these samples some portions corresponding to the ultraviolet absorbing spots on the paper were cut out and extracted with 5 ml. of borate-NaOH buffer (pH 11) and its absorption was measured at maximum peaks by an ultraviolet spectrophotometer. The Rf values and maximum wave lengths are shown in Table I.

Results and Discussion

In studying the excretion rate of metabolites in the rabbit urine, it was recognized that the cyclohexenyl group of EHB was readily oxidized and completely excreted within 10 hours. MHB seemed to be first oxidized in the cyclohexenyl group and then demethylated. In the case of methylbarbital which has no susceptible C-5 side chain, the excretion of barbital, the demethylated compound, was slow but significant. It is evident from the results shown in Figs. 2 and 3 that N-demethylation of these barbiturates is more difficultn than the oxidation of C-5 side group and can be expected when the C-5 group is very resistant to oxidation. These results coincide with results of *in vitro* metabolism as shown in Fig. 4.

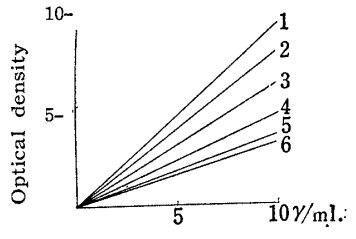


Fig. 1. Calibration Curves of Barbiturates in pH 11 Borate Buffer Solution

1 : 3-keto-nor-MHB 4 : methylbarbital
2 : 3-keto-MHB 5 : MHB
3 : barbital 6 : 3-OH-MHB

8) H. Tsukamoto, Y. Kuroiwa : This Bulletin, **7**, 731 (1959).

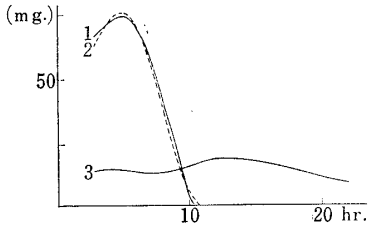


Fig. 2. Excretion Rate of Metabolites of Cyclobarbital and Methylbarbital

1 : 3-OH-EHB
2 : 3-keto-EHB
3 : Barbital

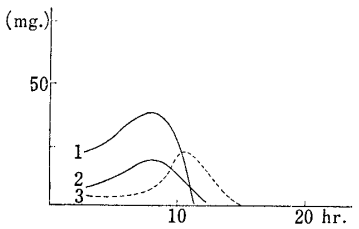
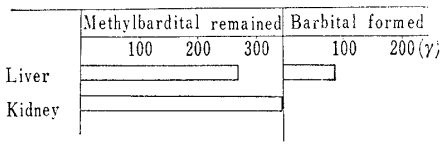


Fig. 3. Excretion Rate of Metabolites of Hexobarbital

1 : 3-OH-MHB
2 : 3-keto-MHB
3 : 3-keto-nor-MHB

1) Methylbarbital by the liver and kidney



2) MHB and its metabolites by the liver

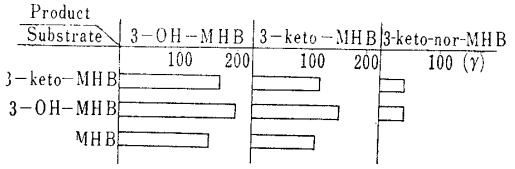


Fig. 4. Demethylation of Barbiturates by Rabbit Tissue Slice

From the fact that the treatment with some liver poisons, partial hepatectomy or complete nephrectomy prolong the duration of anesthesia caused by the administration of various barbiturates, it was suggested[9] that the liver and kidneys play an important role in the detoxication of barbiturate *in vivo*.

In the enzymatic study of MHB, Tsukamoto, *et al.* reported[5] that the microsomal fraction of rabbit liver homogenate could oxidized cyclohexenyl of MHB and yielded 3-keto-MHB and 3-OH-MHB. These two oxidized metabolites were reported to be interconvertible in the soluble fraction. But when they employed these 3-keto and 3-OH-MHB as substrates in the same system, 3-keto-nor-MHB could not be identified as the demethylated compound.

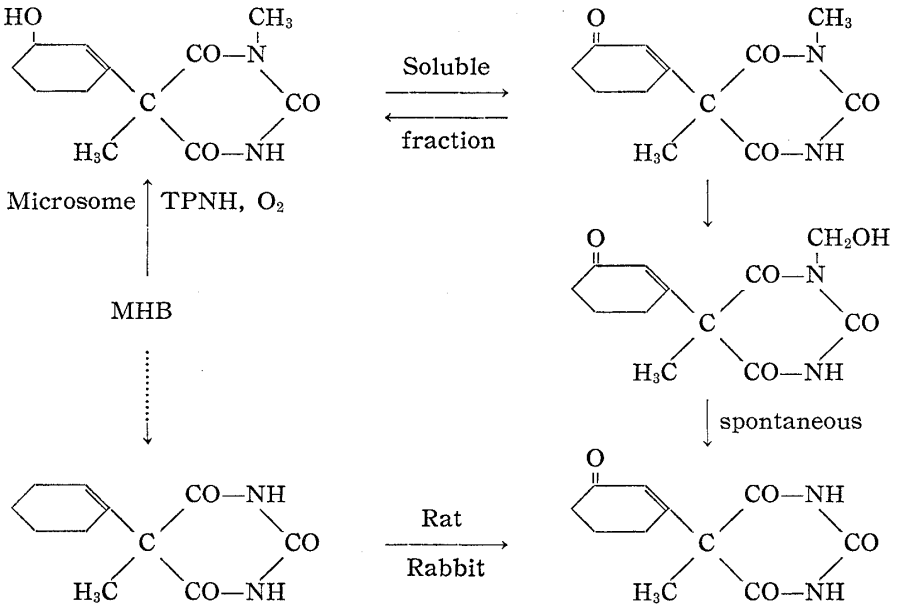


Chart 1.

9) C. M. C. Masson, E. Beland : Anesthesiology, **6**, 483 (1945).

Based on results of the *in vitro* studies shown in Fig. 4, it was found that the liver slice was capable of demethylating MHB and methylbarbital, but that of kidneys was not. Moreover, the production of 3-keto-nor-MHB was observed in the same study only when 3-keto or 3-OH-MHB was used as substrate.

Further investigation will be necessary to obtain the definite evidence, but these findings suggests that, in the metabolism of MHB, the oxidation of cyclohexenyl group takes place initially and is followed by oxidative N-demethylation and that the same enzyme is conducting both the hydroxylation of cyclohexenyl group and N-demethylating reaction in MHB.

Chart 1 is offered for explanation of these observations.

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Summary

The excretion rate of metabolites was investigated *in vivo* after the administration of cyclobarbital, hexobarbital, and methylbarbital in rabbit.

It was found that cyclobarbital was easily oxidized in the cyclohexenyl group and completely excreted within 10 hours. In the case of methylbarbital, the excretion of barbital, the demethylated compound, was slow but significant.

Based on the results of *in vitro* studies it was shown that liver slice was capable of demethylating hexobarbital and methylbarbital, but that of kidneys was not. Moreover, the production of 3-keto-nor-MHB was observed only when 3-keto or 3-OH-MHB was used as the substrate. Therefore, hexobarbital seemed to be first oxidized in the cyclohexenyl group and then demethylated.

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29. Seiichi Okui and Yukio Kuroiwa : Studies on the Metabolic N-Demethylation. II.[1] Barbiturates Induced Acceleration of N-Methylbarbiturates Metabolism.

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In the previous study[1] on *in vitro* metabolism of hexobarital (MHB), it was reported that 3-keto-nor-MHB (5-(3-oxo-1-cyclohexenyl)-5-methylbarbituric acid) was derived from 3-OH-MHB (5-(3-hydroxy-1-cyclohexenyl)-3,5-dimethylbarbituric acid) or 3-keto-MHB (5-(3-oxo-1-cyclohexenyl)-3,5-dimethylbarbituric acid).

The importance of N-demethylation as a mechanism for the *in vivo* inactivation of various N-methylbarbiturates was reported in several papers. Butler, *et al.*[2] synthesized a number of N-substituted derivatives of barbital. N-methylbarbital was found almost completely demethylated in the dog and 69% of the dose appeared in the urine

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1) Part I : This Bulletin, **11**, 160 (1963).

2) T.C. Butler, M.T. Bush : J. Pharmacol. Exptl. Therap., **65**, 205 (1939).