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.

The author is very grateful to Prof. S. Okui, Institute of Pharmacy, School of Medicine, Tohoku University, for his encouragement and to Prof. H. Tsukamoto, Pharmaceutical Institute, Medical Faculty, University of Kyushu, for his kind advice.

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.

(Received February 28, 1962)

UDC 615.782.54-092.21

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).

as barbital. Bush, *et al.*[3,4] isolated phenobarbital from the urine of dogs given prominal (methylphenobarbital). Tsukamoto, *et al.* showed[5,6] that α-, β-OH-MHB, 3-keto-MHB, ureide and 3-keto-nor-MHB were excreted in the urine of rabbits given MHB.

In this paper, the metabolism of MHB, methylbarbital and methylphenobarbital was investigated, especially on demethylation with the rat liver slice pretreated with some barbiturates.

It has recently been found[7~9] that pretreatment of rats with polycyclic hydrocarbon markedly increased demethylation of amino azo dyes and that the similar pretreatment of rats with phenobarbital, barbital, aminopyrine, phenylbutazone, generally increased the activity of enzyme systems which metabolize foreign compounds.

Urethan[10] and chlorcyclizine[11] were reported to have an ability to shorten the duration of hexobarbital hypnosis. Remmer[12] reported that the microsomes of rats treated with phenobarbital oxidized hexobarbital and demethylated methylaminoantipyrine with a much higher rate than those of untreated rats but he did not mention to the demethylation of hexobarbital. In this experiments, phenobarbital, methylbarbital, and barbital were employed for the pretreatment.

Among them, phenobarbital was found to be the strongest accelerant of the metabolism of MHB, methylbarbital and methylphenobarbital. Particularly methylbarbital and methylphenobarbital was rapidly demethylated. In the case of MHB, metabolism itself was undoubtedly increased and oxidation of cyclohexenyl group seemed to be stimulated, but there was no increase in the rate of demethylation. On the other hand, when cyclohexenyl group had already been oxidized, namely in the cases of 3-OH or 3-keto-MHB, phenobarbital treatment could have a significant influence on demethylation reaction.

Experimental

Materials and Methods——Methylphenobarbital was obtained from Dainippon Pharmaceutical Co. Ltd. β-3-OH-MHB was furnished by Tsukamoto Laboratory, Pharmaceutical Institute, Kyushu University. 3-keto-MHB synthesized[13] by chromic oxidation of 3-OH MHB. Methylbarbital[2] was obtained by the methylation of barbital with $(CH_3)_2SO_4$.

Male adult rats weighing 250~300 g. were used for all experiments.

Each 10 mg. of sodium salts of barbiturate, accelerants, dissolved in 0.5 ml. of 0.9% NaCl solution, were given intraperitoneally to a rat for 2 days. Control rats were injected with 0.9% NaCl solution.

Animals were sacrificed 24 hr. after the last injection and the livers were removed. The determination of the ability to metabolize the various barbiturates was carried out in a manner similar to that described previously.[1] The conditions used for the examination of the metabolism of various barbiturates were as follows : 5 μmoles of substrate was incubated 2 hr. with 1 g. of liver slice in Krebs Ringer phosphate buffer which was adjusted to a final volume to 10 ml. The formation of phenobarbital from methylphenobarbital was determined by paper chromatography in combination with ultraviolet absorption spectrophotometric method. Phenobarbital and methylphenobarbital had Rf values of 0.70 and 0.85 respectively, and absorption maxima at 240 and 244 mμ respectively in borate buffer (pH 11).

Determination of Formaldehyde——The degree of enzymatic demethylation of N-methylbarbiturates was determined by estimating the amount of formaldehyde. The formaldehyde generated was trapped

3) M.T. Bush, T.C. Butler : *Ibid.*, **68**, 278 (1940).
4) T.C. Butler : *Ibid.*, **106**, 235 (1952).
5) H. Tsukamoto, H. Yoshimura, S. Toki : This Bulletin, **4**, 368 (1956).
6) H. Yoshimura : *Ibid.*, **5**, 561 (1957).
7) A.H. Conney, J.R. Gillette, J.K. Inscoe, E.C. Trams, H.S. Posner : Science, **130**, 1478 (1959).
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10) J.M. Fujimoto, D.E. Blickenstaff, F.W. Schveler : Proc. Soc. Exp. Biol. Med., **103**, 463 (1960).
11) A.H. Conney, I.A. Michaelson, J.J. Burns : J. Pharmacol. Exptl. Therap., **132**, 202 (1961).
12) H. Remmer : Arch. Exp. Path. u. Pharmakol., **235**, 279 (1959).
13) H. Tsukamoto, H. Yoshimura, S. Toki : This Bulletin, **4**, 364 (1956).

in semicarbazide. A typical reaction mixture was prepared as follows : To a mixture of 4 ml. of 20% homogenate prepared in 1.15% KCl, 100 μmoles nicotinamide, 25 μmoles $MgCl_2$, 100 μmoles neutralized semicarbazide, 5 μmoles substrate in a 50 ml. flask 0.3*M* phosphate buffer solution (pH 7.4) was added to make a final volume of 9 ml. The mixture was incubated in air for 2 hr. at 37°. At the end of incubation period, 1 ml. of 40% CCl_3COOH and 2 ml. of *N* H_2SO_4 were added to the reaction mixture. The formaldehyde was distilled immediately into a graduated tube and 5 ml. of the distillate was collected. To 0.5 ml. of the distillate 4.5 ml. of 0.2% chromotropic acid in 25*N* H_2SO_4 was added subsequently and the formaldehyde was determined spectrophotometrically by the method of MacFadyen.[14]

Table I. Percent MHB and Methylbarbital metabolized by Liver Slices prepared from Pretreated Rats

	Metabolized (%)	
Pretreatment	MHB	Methylbarbital
Phenobarbital	96	52
Methylbarbital	90	41.7
Barbital	81	19.3
Control	70	4.3

Barbiturate in 0.5 ml. of 0.9% NaCl was given to each rat intraperitoneally and the total amount administered for two days was 10 mg. Each enzyme assay was carried out with liver slices from 2 rats. The percentage was calculated from the amount of MHB remained and barbital formed.

Table II. Metabolism of Barbiturates by Liver Slices from Phenobarbital-pretreated rats (μg.)

Substrate \ Metabolite		MHB	3-OH-MHB	3-keto-MHB	3-keto-nor-MHB
MHB	Control	290±10	530±50	222±3	~
	Pretreated	~	760±120	282±38	~
3-OH-MHB	Control		635±74	343±32	10.6±1.3
	Pretreated		610±116	267±73	14.0±2.5
3-keto-MHB	Control		43±15	830±31	37.5±8.3
	Pretreated		102±21	814±9	48.0±9.7

mean±standard deviation

Substrate		Methylbarbital remained	Barbital formed
Methylbarbital	Control	800	44
	Pretreated	306	445

Substrate		Methylphenobarbital remained	Phenobarbital formed
Methylphenobarbital	Control	780	~
	Pretreated	540	139

Male adult rats were injected intraperitoneally with 10 mg. of phenobarbital daily for 2 days. Each incubation flask contained 1 g. of liver slice, 5 μmoles of substrate and sufficient Krebs Ringer phosphate buffer solution to adjust to a final volume of 10 ml. Incubation was carried out for two hours at 37°.

Table III. Percent Demethylation calculated from Formaldehyde Formation

	(%)			
Substrate / Pretreatment	MHB	3-keto-MHB	3-OH-MHB	Methylbarbital
Control	1.2	2.9	8.3	0.5
Phenobarbital	5.1	4.1	3.2	32.0
Methylphenobarbital	2.0	1.7	0.8	21.0
Methylbarbital	1.3	0.3	0.6	9.3
Barbital	1.0			8.0

Rats were administered orally with accelerators (500 μmole/kg.). After 42 hours rats were sacrificed. The liver homogenate was prepared with 1.15% KCl and was assayed for the ability to demethylate MHB, 3-OH-MHB, 3-keto-MHB and methylbarbital. Two animals were used in each group. Percent of substrate metabolized was calculated from the amount of formaldehyde generated.

14) D. A. MacFadyen : J. Biol. Chem., **158**, 107 (1945).

Results and Discussion

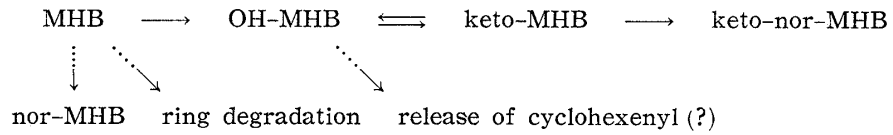
Tables I and II show the rate of the metabolic conversion of N-methylated barbiturates by liver slices prepared from pretreated rats.

As was shown in experimental results, the metabolism of MHB, methylbarbital and methylphenobarbital was obviously accelerated when the animals were pretreated with the three barbiturates.

The liver slices prepared from phenobarbital pretreated rats could oxidize the cyclohexenyl group of MHB in an extent of 1.3~1.6 times than of the untreated preparation. Although this pretreatment elevated the activity of liver slice to demethylate methylbarbital up to 12 times, 3-keto-nor-MHB could not be detected in the metabolites of MHB. Both 3-OH-MHB and 3-keto-MHB have a little tendency to be converted to 3-keto-nor-MHB.

Total recovery of metabolites from 3-OH-MHB seemed to be lower in pretreated than in control. It could have been caused by other unknown pathways such as ring degradation, release of cyclohexenyl group,[15] etc.; these possibilities should be confirmed by further investigation. Table III shows that a less amount of formaldehyde is detected from 3-keto-MHB and 3-OH-MHB than that released from MHB and methylbarbital. It seems probable that the difference between the results obtained from the paper chromatographic method and formaldehyde analysis is due to the semicarbazone formation of 3-keto-MHB and oxidized 3-OH-MHB. This result suggests the improbable occurrence of direct demethylation of MHB and 3-OH-MHB besides the main pathway presented in a previous report.[1]

It is of interest in the metabolism of 3-keto-MHB in the rat liver that the equilibrium between 3-keto-MHB and 3-OH-MHB in the metabolite goes far to fore follow-3-keto-MHB. This is markedly different from that found with the rabbit liver. Thereing steps can be assumed to occur in the rat liver :



In the metabolism of methylbarbital and methylphenobarbital, which possess a C-5 side group stable to oxidation, the pretreatment led the compounds to the pathway of demethylation rather than to that of the oxidation of C-5 substituted group as described above.

Brodie suggested[16] that the oxidative pathway in microsomal fraction was entirely through hydroxylation reaction, that is, the direct substitution of hydroxyl grouping for a hydrogen.

The results obtained in the present experiments were also compatible with the hypothesis presented in a previous paper, that is, in the metabolism of MHB, the oxidation of cyclohexenyl group occurs initally and is followed by oxidative N-demethylation, both hydroxylation and demethylation of MHB being conducted by the same enzyme system, and the degree of susceptibility depends only on the chemical stability of each group to oxidative reaction.

The authors are indebted to Prof. H. Tsukamoto, Kyushu University, for his kind supply of 3-OH-MHB and also to Mr. K. Koike for his assistance in a part of this work.

A part of expense of this work was financed from Grant-in-Aid for Scientific Research from the Ministry of Education, to which author's thanks are due.

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16) B. B. Brodie, J. R. Gillette, B. N. LaDu : Annu. Rev. Biochem., **27**, 427 (1958).

Summary

The metabolism of hexobarbital, methylbarbital and methylphenobarbital was investigated from the viewpoint of demethylation using the liver slice of rats pretreated with barbiturates such as phenobarbital, methylbarbital and barbital. phenobarpital had the strongest effect in accelerating the metabolism of all substrates.

Methylbarbital and methylphenobarbital, both which possess the C-5 side group stable to oxidation, were led by the pretreatment to the pathway of demethylation rather than to that of oxidation of C-5 substituted group.

Although the oxidation of cyclohexenyl group in the metabolism of hexobarbital was stimulated by the pretreatment, no demethylated metabolite could be detected. When the cyclohexenyl group of substrate was oxidized beforehand, demethylation could take place and was promoted by the pretreatment.

Based on these findings, the relationship between hydroxylation and demethylation of N-methylbarbiturates was discussed.

(Received February 28, 1962)

UDC 615.32:582.736

30. Shoji Shibata and Yoshihiro Nishikawa*1 : Studies on the Constituents of Japanese and Chinese Crude Drugs. VII.*2 On the Constituents of the Roots of *Sophora subprostrata* Chun et T. Chen, (2),[1] and *Sophora japonica* L. (1).

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In the previous paper,[1] on the constituents of the root of *Sophora subprostrata* Chun et T. Chen (Chinese Drug : Shan-Dou-Gen (山豆根)) we reported the presence of two unknown neutral substances, $C_{22}H_{22}O_{10}\cdot CH_3OH$ and $C_{17}H_{14}O_5\cdot \frac{1}{2}H_2O$, tentatively named B_1 and C_1, along with some alkaloids, matrine, oxymatrine, anagyrine and methylcytisine.

On surveying the analogous constituents in other Sophora plants, we have studied the principles of the root of *Sophora japonica* L.

The constituents of the ground part of this plant were investigated earlier, and it was reported that the following compounds were isolated : Rutin,[2] sophoradiol[3,4] and betulin,[3] from the flower buds, and rutin,[5] quercetin[5] sophoflavonoloside,[6] sophoricoside,[7] and sophorabioside,[8] from the fruits.

*1 Bunkyo-ku, Hongo, Tokyo (柴田承二, 西川嘉広).

*2 Part VI : S. Shibata, T. Murata : Yakugaku Zasshi, **82**, 777 (1962).

1) (1) : S. Shibata, Y. Nishikawa : *Ibid.*, **81**, 1635 (1961).
2) *a*) W. Stein : J. Proc. Chem. Soc., **58**, 399 (1853); *b*) E. Schmidt : Arch. Pharm., **242**, 216 (1904); *c*) Y. Shibata, K. Kimotsuki : Acta. Phytochim (Tokyo), **1**, 97 (1923).
3) T. Kariyone, S. Ishimasa, T. Shiomi : Yakugaku Zasshi, **76**, 1210 (1956).
4) *a*) K. Kimura, M. Takahashi, S. Ishimasa, Y. Kodama : *Ibid.*, **78**, 1090 (1958). *b*) S. Ishimasa: *Ibid.*, **80**, 304 (1960).
5) Y. Hirose : *Ibid.*, **28**, 227 (1909).