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238. Yukio Kuroiwa, Ken-ichiro Minegishi, and Seiichi Okui : Studies on the Metabolic N-Demethylation. III.*2 Acceleration of Drug Metabolizing Enzyme in Rat Liver.

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In recent years, it has recognized that activity of drug metabolizing enzymes in liver microsomes can be influenced by the administration of foreign compounds, hormones and nutritional states of animals.

Many workers reported[1~6] that administration of foreign compounds such as phenobarbital, 3-methylcholanthrene (3-MC) etc., markedly increase the activity of several enzyme systems in rat liver microsomes which require NADPH (reduced nicotinamide adenine dinucleotide phosphate) and oxygen as cofactors.

A lot of reactions which are catalyzed by these enzyme systems were described so far. Among them a few reactions are stimulated by the administration of particular accelerants, so it is not unlikely that oxidative drug metabolizing enzymes mentioned above will be classified to several groups from the aspect of activation.

In this paper the demethylation of N-methylbarbiturates was investigated using the liver of phenobarbital-treated rat and it was found that this reaction also required NADPH and oxygen but apparently differed from the demethylation of aminoazo dyes which is known to be stimulated by polycyclic hydrocarbons.

Further, the change of activity of drug metabolizing enzyme against the several substrates was traced after the administration of phenobarbital or 3-MC. The results suggested that the demethylation of methylbarbital and hydroxylation of cyclobarbital were conducted by the same kind of enzyme system, but the hydroxylation of acetanilide seemed to be conducted by different one from this system.

Experimental

Materials and Methods——Glucose-6-phosphate and NADP were obtained from the Nutritional Biochemicals Corp. NADPH was prepared chemically by the method of Kaplan, *et al.*[7] and Hamm, *et al.*[8] Hexobarbital and cyclobarbital were obtained from the Dainippon Pharmaceutical Co. Ltd. and Shionogi Co., & Ltd. Monomethylaminoazobenzene (MAB) was furnished by Dr. A. Hanaki of National Institute of Radiological Science (Japan) and benzpyrene by Dr. Y. Masuda of Department of Hygiene, Kyushu University and 3-methyl-MAB by Prof. Z. Tamura, University of Tokyo. 3-MC was obtained from the Wako Pure Chemical Industries Ltd. Methylbarbital was synthesized by the method of Butler.[9]

Male wister rats weighing from 150 to 200 g. were used to supply liver tissue. Experimental groups were pretreated as follows : Sodium salts of barbiturates were given orally in 1 ml. of 0.9% NaCl solution in a dose of 500 μ moles/kg. DAB (dimethylaminoazobenzene) was suspended in a 5% acacia solution and administered in a dose of 20 mg. per rat. Benzpyrene and 3-MC dissolved in 0.3 ml. of cotton seed oil were given intraperitoneally in doses of 2 mg. and 5 mg. per rat respectively.

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Animals were killed 24 hr. after the administration of drugs by a blow on the head then decapitated. The livers were immediately removed and homogenized in four parts of 0.1*M* phosphate buffer (pH 7.4) with Potter Elvehjem type homogenizer. The homogenate was then centrifuged at 9000×g for 15 min. and the supernatant fraction poured out and stored in ice H_2O until used. The 78000×g soluble fraction and microsomal fraction were prepared by differential centrifugation of the homoge nate as described by Cooper and Brodie.[10]

A typical incubation mixture contained 4 ml. of enzyme preparation, 100 μ moles of nicotinamide, 50 μ moles of $MgCl_2$, 5 μ moles of substrate made up to final volume of 10 ml. with 0.1*M* phosphate buffer. MAB or 3-methyl-MAB as the substrate was dissolved in 0.2 ml. of 70% EtOH and used.

The determinations for ability to metabolize the hexobarbital, methylbarbital and cyclobarbital were performed similar to that described previously.[11] Sometimes cyclobarbital was assayed by the method of Takabatake.[12] 4-Hydroxyacetanilide was extracted and assayed as described by Krisch, *et al.*[13]

The degree of the enzymatic demethylation of MAB, 3-methyl-MAB and methylbarbital was determined by estimation of the amount of HCHO.*1 The HCHO generated was trapped with semicarbazide. At the end of the incubation period, 1 ml. of 30% CCl_3COOH and 1 ml. of *N* H_2SO_4 were added to the reaction mixture. Reaction mixture was centrifuged to remove protein and the supernatant was poured into distillating flask. The HCHO was distilled immediately into a graduate tube. Aliquot was taken from distillate and the HCHO was determined with chromotropic acid method.

Results

I. Requirement of NADPH for the Metabolic Demethylation of Hexobarbital and Methylbarbital by Pretreated Rat Liver Microsomes

As shown in Table I, the activity of 9000×g supernatant preparation almost disappeared when dialysed against the deionized water at 0~4° for 24 hour. By the addition of NADP and glucose-6-phosphate which generate NADPH, the activity was restored.

Table I. Effect of NADP on the Demethylation of Methylbarbital by the Dialysed 9000×g Supernatant Fraction (PS)

Addition	HCHO generated (μ mole)
PS + NADP + glucose-6-phosphate	1.26
PS + NADP	0.01
PS + NADPH	0.40
PS	0.00

The dialysed 9000×g supernatant derived from 1 g. of rat liver which was pretreated with intraperitoneal injection of phenobarbital (10 mg./rat) for two days was incubated for 2 hr. at 37° in medium containing 100 μ moles of nicotinamide, 25 μ moles of $MgCl_2$, 2.5 μ moles of methylbarbital, 1 mg. of glucose-6-phosphate, 2 μ moles of NADP and 0.1*M* phosphate buffer (pH 7.4). Final volume 10 ml.

Microsomes and soluble fraction were prepared by 78000×g centrifugation of 9000×g liver supernatant. The demethylated hexobarbital and methylbarbital was detected by paper chromatography as reported previously.[11] As indicated in Table II, enzyme activity of microsomes was appeared by the addition of NADPH.

II. Distribution of Enzyme Activity in the Pretreated Liver Cell

Distribution of enzyme activity in liver cell which was pretreated with benzpyrene and phenobarbital was showed in Table III. This result indicated that the high enzyme activity to oxidize hexobarbital and to demethylate methylbarbital were only responsible for the microsomal fraction from the phenobarbital-treated rat and that there exist no activator in the soluble fractions. It seemed that in the benzpyrene-treated rat the oxidation of hexobarbital was slightly decreased than the control rat.

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Table II. Demethylation Activity of Microsomes and Soluble Fraction of the Pretreated Rat Liver

	3-Keto-nor-MHB[a] formed (μg.)	Barbital formed (μg.)
Microsomes	<0.1	<0.1
Soluble fraction	~	~
Microsomes + soluble fraction	30	416
Microsomes + NADPH	10	65

Microsomes and soluble fraction from 1 g. of rat liver which was pretreated with intraperitoneal injection of phenobarbital (10 mg./rat) for two days were incubated for 2 hr. in medium containing 100 μ moles of nicotinamide, 25 μ moles of $MgCl_2$ and 5 μ moles of 3-OH-MHB[a] or methylbarbital as substrate. The addition of NADPH, in all 2 μ moles, were carried out at 30 min. interval.

Amount of demethylated compounds were estimated by the method of paper chromatography as described previously.[11]

a) Abbreviations used : 3-keto-nor-MHB, 5-(3-oxo-1-cyclohexenyl)-5-methylbarbituric acid. 3-OH-MHB, 5-(3-hydroxy-1-cyclohexenyl)-3,5-dimethylbarbituric acid.

Table III. Localization of Enzyme Activity in the Rat Liver which was pretreated with Phenobarbital or Benzpyrene

Microsomes	Soluble fraction	HCHO formed from methylbarbital (μ mole)	MHB remained (μg.)	3-OH-MHB formed (μg.)	3-Keto-MHB[a] formed (μg.)
Control	control	0.13	735	180	165
	benzpyrene	0.13	750	172	151
	phenobarbital	0.16	725	184	133
Benzpyrene	control	0.16	883	172	105
	benzpyrene	0.13	940	135	74
	phenobarbital	0.19	940	149	91
Phenobarbital	control	0.87	662	221	196
	benzpyrene	0.70	650	214	161
	phenobarbital	0.87	645	218	183

Each incubation flask contained 4 ml. of enzyme solution prepared from 1 g. liver, 100 μ moles of nicotinamide, 50 μ moles of $MgCl_2$, 2.5 μ moles of methylbarbital or 5 μ moles of hexobarbital (MHB), and 0.1*M* phosphate buffer to a final volume of 10 ml. Incubation was carried out for 2 hr. at 37°.

a) 3-keto-MHB, 5-(3-oxo-1-cyclohexenyl)-3,5-dimethylbarbituric acid.

III. The Effect of Carcinogenic Substances and Barbiturates for the Demethylating Enzyme System

In order to clarify the effect of carcinogenic substances and barbiturates on the demethylating enzyme system, the rats were pretreated with benzpyrene, 3-MC and DAB as carcinogenic substance and phenobarbital, methylbarbital and cyclobarbital as barbiturate. To observe the hydroxylation of side chain and phenyl group, cyclobarbital and acetanilide were used as substrates and to observe the demethylation of N-methyl group, methylbarbital and MAB were employed.

As shown in Table IV, V, demethylation of MAB was activated not only carcinogenic substances but also phenobarbital and some other barbiturates. on the other hand, the carcinogenic substances are active for the demethylation of MAB but inactive for the demethylation or hydroxylation of barbiturates.

IV. Increasing Activity of the Enzyme System after the Administration of 3-MC and Phenobarbital

Fig. 1 show the alteration of inductive effect after the administration of 3-MC and phenobarbital. It was observed that there appeared two maximum peaks around 24~40 and 60~70 hours afters pretreatment.

Table IV. Effect of Drugs on the Demethylation

Drugs pretreated	HCHO generated (μ mole)	
	from MAB	from methylbarbital
Control	0.23	0.12
3-MC	0.40	0.10
Benzpyrene	0.57	0.09
DAB	0.80	0.07
Phenobarbital	0.70	1.20
Methylbarbital	1.00	0.91
Cyclobarbital	0.50	0.32

9000×g supernatant corresponding to 1 g. of liver was used. Each incubation medium were contained 50 μ moles of $MgCl_2$, 100 μ moles of nicotinamide, 100 μ moles of semicarbazide and 2.5 μ moles of MAB or methylbarbital. Incubation time was 1 hr. for MAB and 2 hr. for methylbarbital.

Table V. Effect of Drugs on the Metabolism of Cyclobarbital (EHB) and Acetanilide

Drugs pretreated	Acetanilide hydroxylated (μ mole)	EHB remained (μg.)	3-OH-EHB[a] formed (μg.)	3-keto-EHB[a] formed (μg.)
Control	1.2	730	197	38
3-MC	2.5	835	180	27
Benzpyrene	1.6	805	181	24
DAB	1.1	924	172	33
Phenobarbital	1.4	610	363	57
Methylbarbital	1.2	622	272	74
Cyclobarbital	1.2	665	263	37

Each incubation medium were contained 50 μ moles of $MgCl_2$, 100 μ moles of nicotinamide, 5 μ moles of substrate and 4 ml. of 9000×g supernatant. Incubation time was 1 hr. for acetanilide and 2 hr. for EHB.

a) 3-OH-EHB, 5-(3-hydroxy-1-cyclohexenyl)-5-ethylbarbituric acid. 3-keto-EHB, 5-(3-oxo-1-cyclohexenyl)-5-ethylbarbituric acid.

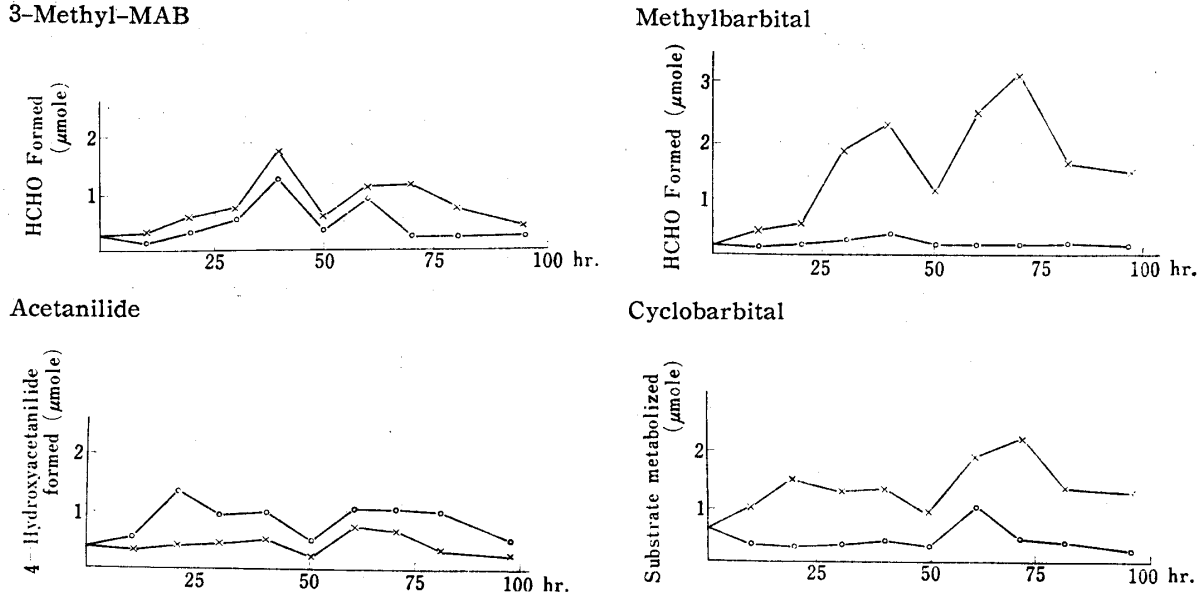

Fig. 1. The Alteration of Inductive Effect after the Administration of 3-MC and Phenobarbital

×—×— Pretreated by phenobarbital
○—○— Pretreated by 3-MC

When 3-methyl-MAB was used as the substrate after the pretreatment of 3-MC and phenobarbital, the time course of the demethylation activity showed quite similar process, but demethylation of methylbarbital was not affected by 3-MC.

Therefore, the results suggest that there are at least two enzyme systems responsible for demethylation.

After the administration of phenobarbital, the hydroxylation of acetanilide was not effected during the first 50 hours but the hydroxylation of cyclobarbital was markedly increased as well as the demethylation of methylbarbital. On the contrary, the hydroxylation of acetanilide was increased by the administration of 3-MC but the hydroxylation of cyclobarbital was not stimulated.

Consequently it was considered that there were also at least two enzyme systems for hydroxylation and the hydroxylation of barbiturate may be closely related to the demethylation of methylbarbiturate. The inductive effect which appeared during 60~70 hours after the pretreatment may be considered secondary nonspecific activation.

Discussion

In earlier papers many workers reported the oxidative detoxication of a number of foreign compounds by liver microsomes in the presence of NADPH and oxygen. The demethylation of N-methylbarbiturates also required the same cofactor, since the activity of 9000×g supernatant which was dialysed against the deionized water was restored by the addition of both NADP and glucose-6-phosphate.

This reaction was considerably accelerated with phenobarbital pretreatment. The stimulatory effect was not observed when excess cofactors were added to the enzyme prepared from untreated animal, so such acceleration appeared to be obliged to enzyme biosynthesis.[14]

Conney, *et al.*[14] showed that the administration of benzpyrene or 3-MC caused little or no stimulation of liver microsomal enzymes, *i.e.* on the hydroxylation of chlorzoxazone, N-demethylation of aminopyrine and oxidation of hexobarbital. Posner, *et al.*[15] and Creaven, *et al.*[16] have suggested that the hydroxylation reaction seemed to be conducted in liver microsomes under the variety of enzyme systems, therefore the enzyme system for the N-demethylation of aminoazo dyes are expected to be different from the system for the N-demethylation of aminopyrine.

In the experiment on the demethylation of methylbarbital the pretreatment of benzpyrene did not enhanced the activity to demethylate methylbarbital as well as to hydroxylate cyclobarbital. Accordingly the enzyme system which differed from the demethylation of aminoazo dyes seems to be conducting both the hydroxylation of cyclobarbital and N-demethylation of methylbarbital.

Both demethylation of methylbarbital and hydroxylation of cyclobarbital were apparently depressed by the pretreatment of carcinogenic substances but demethylation of MAB was stimulated with both carcinogenic substances and barbiturates.

From this observation, it is considered that there were at least two enzyme systems which required NADPH and oxygen for the demethylation reaction.

In order to ascertain these results in the viewpoint of inductive effects, increase of activity after the administration of 3-MC and phenobarbital was investigated.

Based on results shown in Fig. 1, it was found that in the course of activation by pretreatment of phenobarbital, two maximum points were observed around 24~40 hours

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and 60~70 hours when used the cyclobarbital, methylbarbital and 3-methyl-MAB as the substrates, but demethylation of methylbarbital differed from the demethylation of 3-methyl-MAB when pretreated by 3-MC.

Although further investigation will be necessary to obtain the definite evidence, these findings suggest that there are close relationship between demethylation and hydroxylation of barbiturates and that the one peak may be occurred by the direct induction of pretreated drugs, and the other may be induced by the influence of drug metabolites or the hormonal effect which is affected with drugs.

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Summary

The demethylation activity for the N-methylbarbiturates was localized in the microsomal fraction and required NADPH and oxygen. This activity was increased markedly with pretreatment of some barbiturates but not with carcinogenic substances such as DAB, 3-MC and benzpyrene.

The activity of the demethylation or hydroxylation of barbiturates was generally decreased by the pretreatment of carcinogenic substances.

In accord with the increase of enzyme activity, in the time course of induction after the pretreatment of phenobarbital, two maximum peaks were appeared around 24~40 hours and 60~70 hours when cyclobarbital, methylbarbital, and 3-methyl-MAB were employed as the substrates. By the pretreatment of 3-MC the demethylation of 3-methyl-MAB was stimulated, but the demethylation of methylbarbital was not.

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