[Chem. Pharm. Bull. 13(6) 731~734 (1965)] UDC 615.7-092.21

98. Yukio Kuroiwa, Ken-ichiro Minegishi, and Seiichi Okui : Studies on the Metabolic N-Demethylation. IV. Effects of Phenobarbital and Morphine on the Oxidative Demethylation in Rat Liver.

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It has been shown that various drugs[1~8] can be oxidized by liver microsomal fraction which requires molecular oxygen and NADPH (reduced nicotinamide adenine dinucleotide phosphate) as cofactors, and that the activity of the microsomal fraction can be influenced by the administration of foreign compounds and hormones, and by the nutritional states of animals.

Especially, the administration of foreign compounds such as phenobarbital, 3-methylcholanthrene, etc., significantly increases the activity of drug metabolism in liver microsomal fraction. Previously we have shown the elevation of oxidative demethylation of methylbarbital caused by the administration of phenobarbital. This stimulative activity of phenobarbital is denoted as phenobarbital-effect in this paper. On the other hand, some workers[9~10] have suggested that the repeated administration of morphine reduces oxidative demethylation of narcotics.

In this paper, it was described that the influence of repeated administration of morphine on the magnitude of phenobarbital-effect and that the incorporation of ^{14}C-labeled amino acids in such condition.

The results suggested that the administration of morphine might produce inhibition of drug metabolism, and that the incorporation of ^{14}C-amino acids into microsomal proteins was apparently increased in phenobarbital treated rats and that morphine itself did not affected on the incorporation rate of ^{14}C-amino acids.

Experimental

Materials——Morphine, meperidine, codeine and phenobarbital were commercially obtained. Methylbarbital was synthesized by the method of Butler, *et al.*[11] ^{14}C-Amino acids (chlorera hydrolysate) was obtained from Daiichi Co. Ltd.

Determination of Enzyme Activity——Experimental details were as follows. Five male wister rats of each group weighing 180~230 g. were used to supply liver tissue. Sodium salts of phenobarbital was given orally in 1 ml. of 0.9% NaCl solution in a dose of 500 μmoles per kg. body weight. Morphine hydrochloride dissolved in the dose of 100 mg. per kg. body weight.

Animals were decapitated 24 hr. after the administration of drug. The livers were immediately removed and homogenized in 4 volumes of 1.15% KCl solution with Potter-Elvehjem type homogenizer. The homogenate was then centrifuged at 9,000×g. for 30 min. and the supernatant fraction was obtained and stored in ice cold until use.

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A typical reaction mixture of oxidative demethylation, previously reported,[8] containing 4 ml. of 9,000 ×g. supernatant, 100 μmoles of nicotinamide, 100 μmoles of semicarbazide, 50 μmoles of $MgCl_2$, 5 μmoles of substrate and 1 ml. of 0.3*M* phosphate buffer (pH 7.4) was made up to final volume of 10 ml. with distilled water, and incubated for 2 hr. at 37°.

The activities of the oxidative demethylation of the meperidine, codeine, morphine and methylbarbital were determined by estimation of the amount of HCHO formed which 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 removed protein. The supernatant was poured into distillating flask and HCHO was distilled immediately into a graduate tube. An aliquot was taken from distillate and the amount of HCHO was determined with chromotropic acid method.[12]

Incorporation of ^{14}C-Amino Acids——^{14}C-Amino acids (2 μc.) was administered intraperitoneally 2 hr. prior to the sacrifice. Rats were sacrificed by decapitation and the livers were rapidly removed into cold 0.25*M* sucrose solution, weighed, and homogenized in 9 volumes of Medium A[13] (0.25*M* sucrose, 0.025*M* KCl, 0.005*M* $MgCl_2$ and 0.05*M* Tris buffer, pH 7.4) with Potter-Elvehjem type homogenizer in an ice bath.

A nuclei and cell debris fraction was removed by centrifuging at 700×g. for 10 min. A mitochondrial fraction was sedimented from 700×g. supernatant fluid at 8,000×g. for 10 min. A microsomal fraction was sedimented by centrifugation of the 8,000×g. supernatant at 105,000×g. for 60 min. A soluble fraction was obtained from 105,000×g. supernatant by addition of cold 6*N* perchloric acid.

Each fraction, except the nuclei and cell debris fraction, was washed three times with cold 0.5*N* perchloric acid, and treated with alcohol-ether-chloroform (2:2:1) to remove lipids. Proteins were dissolved with 1*N* NaOH, kept for 1 hr. at room temperature, and again precipitated with 6*N* HCl and washed three times with cold 0.5*N* perchloric acid. The nuclei and cell debris fraction was three times with cold 0.5*N* perchloric acid at 70° for 15 min. to remove deoxyribonucleic acid and treated described above.

An aliquot of each protein fraction was dissolved in 3 ml. of 10% NH_4OH. One ml. of each sample was placed on a planchet and dried under a heat lamp, and the radioactivity was counted by gas-flow counter. The protein content of each sample was determined by Biuret method.[14]

Results

I. Increased Activity of the Oxidative Demethylation in Rat Liver Pretreated with Phenobarbital

In this experiment, Table I shows the altered activities of oxidative demethylation after administration of phenobarbital. Meperidine, N-methylbarbital, morphine, and codeine (O-methyl-compound) were used as substrates. In phenobarbital treated rats,

Table I. Increased Activity of the Oxidative Demethylation in Rat Liver Pretreated with Phenobarbital

Substrate	Metabolized (%)		Ratio of activation
	nontreated	treated	
Methylbarbital	6.8±1.8	42±9	6.19
Meperidine	84 ±7	90<	<1.19
Codeine	70 ±5	82±6	1.17
Morphine	33 ±4	34±6	1.06

$$\text{ratio of activation} = \frac{\text{whole activity after pretreatment}}{\text{control activity}}$$

$$\text{metabolized } \% = \frac{\text{metabolite}}{\text{substrate}} \times 100 \quad \text{(mean value±standard error)}$$

Each rat was administered orally 500 μmoles phenobarbital per kg. body weight at 24 hr. prior to the sacrifice.

Each reaction mixture containing 4 ml. of 9,000×g. supernatant, 100 μmoles of nicotinamide, 100 μmoles of semicarbazide, 50 μmoles of $MgCl_2$, 5 μmoles of substrate and 1 ml. of 0.3*M* phosphate buffer (pH 7.4) was made up to final volume of 10 ml. with distilled water, and incubated for 2 hr. at 37°.

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methylbarbital was demethylated as much as six times of that of control rats. Meperidine is easily metabolized in control rats, but the ratio of activation is not so high. Codeine was metabolized in the same extent, and in the case of morphine no significant difference was found out between control rats and treated rats.

II. Effects of Morphine on the Oxidative Demethylation in Rat Liver

As shown in Fig. 1, on three substrates, except methylbarbital, the activity of demethylation declined remarkably during first four days and lowered gradually afterward. There was no significant difference between control rats and morphinized rats, since methylbarbital was only slightly even in normal rats.

III. Effects of Phenobarbital on the Oxidative Demethylation in Morphinized Rat Liver

Effects of phenobarbital on the oxidative demethylation in rat liver which was pretreated with 100 mg. of morphine per kg. body weight for four days was shown in Fig. 2. Though the activity of the oxidative demethylation in morphine treated rats was reduced markedly, the effects of activation with phenobarbital was remained.

But, after successive injection of morphine for a month, the effects of methylbarbital was found to be diminished almost completely, while the demethylating activity against meperidine was still remained.

IV. The Incorporation of ^{14}C-Labeled Amino Acids into Morphinized Rat Liver *In Vivo*

The relation between protein synthesis and demethylating enzyme activity was tested in this experiment. As shown in Fig. 3, the results showed that incorporation of ^{14}C-amino acids into microsomal protein was significant increased than other fractions by the administration of phenobarbital. But morphine itself did not show any effects on the normal incorporation rate of amino acids into these fraction. After the successive injection of morphine, the effects of phenobarbital on the incorporation of amino acids into four fractions of morphinized rats appeared to be rather inhibited than that of control rats.

Discussion

In this paper, phenobarbital has been considered as an activator of microsomal detoxicating enzymes. Based on results shown in the Table I, methylbarbital was

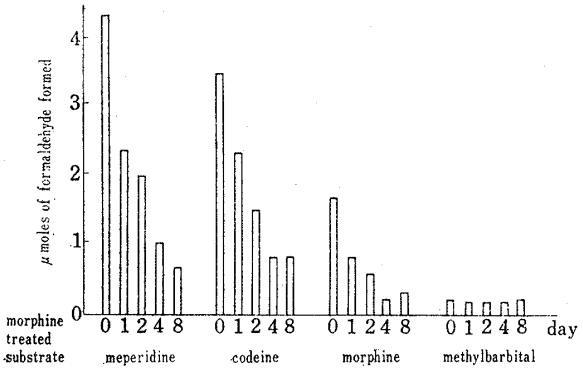

Fig. 1. Effects of Morphine on the Oxidative Demethylation in Rat Liver

In the above conditions, rats were administered intraperitoneally 100 mg. of morphine-hydrochloride per kg. body weight, and were sacrificed 24 hr. after the last injection. Control rats were similarly treated with 0.9% saline.

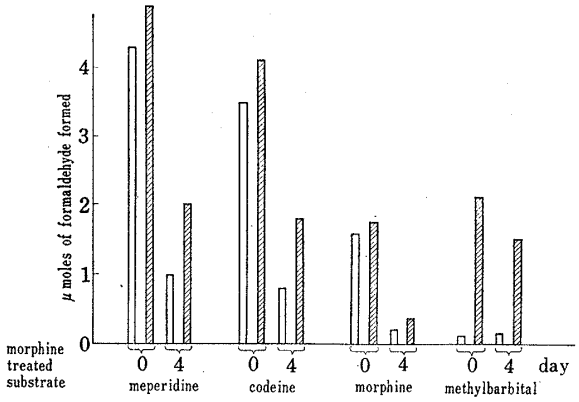

Fig. 2. Effects of Phenobarbital on the Oxidative Demethylation in Morphinized Rat Liver

□ control. ▨ phenobarbital treated.
Rats were administered intraperitoneally 100 mg. of morphine-hydrochloride per kg. body weight for four days, and were administered orally 500 μmoles of phenobarbital per kg. body weight at 24 hr. prior to the sacrifice. Control rats were treated with 0.9% saline.

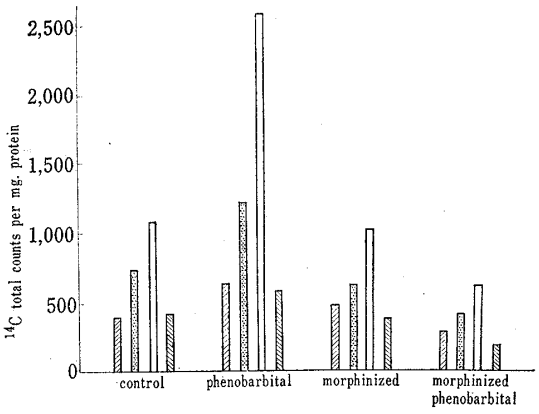

Fig. 3. The Incorporation of ^{14}C-Labeled Amino Acids into Morphinized Rat Liver *In Vivo*

▨ nucleus and cell debris.
▩ mitochondria.
□ microsomes.
▩ soluble protein.

Rats were morphinized 4 weeks as indicated below, 1st week 15 mg. per kg., 2nd week 30 mg. per kg., 3rd week 45 mg. per kg. and last week 60 mg. per kg. body weight.

Rats were administered orally 500 μmoles of phenobarbital per kg. body weight 24 hr. and intraperitoneally ^{14}C-amino acids (2 μc.) 2 hr. prior to the sacrifice respectively.

The results given represent averages obtained from at least 3 determinations.

slightly metabolized in control rats whereas was remarkably metabolized in phenobarbital treated rats. It is considered that methylbarbital is very convenient to the phenobarbital-effect as a substrate, on the other hand, meperidine is useful to measure the degree of inhibition in enzyme activity by narcotics such as morphine etc.

In our results illustrates in Fig. 1 and Fig. 2, the demethylation of meperidine was markedly inhibited in the earlier period of morphine treatment. The response to phenobarbital indicates a sort of biological response or, in other words, a sort of adaptation, and the response to the drugs such as phenobarbital.

Further, it was represented in Fig. 3 that the effect of phenobarbital on the incorporation of ^{14}C-amino acids into chronic morphinized rats was depressed than that into the control rats.

But morphine itself did not affected on the incorporation of ^{14}C-amino acids into morphinized rats.

As mentioned above, it is likely that phenobarbital has nonspecific action on the both demethylating enzyme activities and incorporation of ^{14}C-amino acids. Morphine acts on the inhibition of demethylating enzyme system and diminishes the effect of activation with phenobarbital. The nature of this effect of morphine is now under investigation.

Summary

It is very convenient to use methylbarbital as substrate in order to check the phenobarbital-effect, and on the other hand, meperidine is useful to measure the degree of inhibition in the enzyme activity by narcotics such as morphine etc.

It was recognized that the repeated administration of morphine to rats caused gradually depression of detoxicating enzyme activity in demethylation of narcotics, but did not affect on the incorporation of ^{14}C-amino acids into rat liver protein.

(Received March 12, 1965)