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Studies on the Metabolic N-Demethylation. V.[1] Effect of Phenobarbital on the Oxidative Demethylation *in Vitro* in the Liver of Adrenalectomized Rats

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The activities of demethylating enzymes were decreased by adrenalectomy, but were stimulated by the administration of phenobarbital or cortisone in the liver of adrenalectomized rats. Incorporation of ^{14}C-labeled amino acid into liver microsomal proteins of adrenalectomized rats was apparently increased by the administration of phenobarbital. The magnitude of phenobarbital-effect was decreased by pretreatment with puromycin and 8-azaguanine in intact animals.

It has been recognized that physiological and artificial factors which alter the activity of hepatic microsomal enzyme systems include stress, administration of some hormones, feeding with foreign compounds, and nutritional state of the animal.[4] However, no detailed information has been obtained about the relationship between drug-metabolizing enzymes and such factor, especially concerning adrenal function.

The experiments described in this paper were conducted to examine the effect of phenobarbital on the microsomal drug-metabolizing enzymes in rats with or without adrenalectomy and influence of cortisone.

Experimental

Materials and Methods——Morphine hydrochloride, meperidine hydrochloride, sodium phenobarbital, and cortisone acetate were commercially obtained. Methylbarbital was synthesized by the method of Butler and Bush.[5] Puromycin was furnished by the National Institute of Health, Tokyo, and 8-azaguanine from the Takeda Chemical Industries, Ltd.

Animals and Treatment——Five male Wistar rats, weighing 110—150 g were used to supply liver tissue. Animals were bilaterally adrenalectomized and maintained on 0.9% saline. Studies were made 3 days after adrenalectomy. Phenobarbital was given orally in 1 ml of 0.9% saline in a dose of 500 μmoles/kg

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2) Deceased March 18, 1967.
3) Location: *Kita-4-bancho, Sendai.*
4) A.H. Conney and J.J. Burns, *Advance in Pharmacol.*, 1, 31 (1962).
5) T.C. Butler and M.T. Bush, *J. Pharmacol. Exptl. Therap.*, 65, 205 (1939).

24 hr before sacrifice unless otherwise indicated. Cortisone acetate was given intraperitoneally in doses of 5 or 25 mg/kg/day for 3 days. Puromycin dissolved in 0.5 ml of 0.9% saline was given intraperitoneally in a dose of 10 mg/kg four times every 2 hr before sacrifice. 8-Azaguanine dissolved in 0.5 ml of 0.9% saline was given intraperitoneally in a dose of 100 mg/kg three times every 15 hr before sacrifice.

Animals were decapitated and the livers were immediately removed and homogenized in 4 volumes of 1.15% KCl solution in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 9000 *g* for 30 min and the supernatant fraction was stored in an ice cold bath until use.

Determination of Enzyme Activities——Activities for the oxidative demethylation of meperidine, morphine, and methylbarbital were determined by estimation of the amount of formaldehyde formed which was trapped with semicarbazide. A typical reaction mixture of oxidative demethylation, previously reported,[6] containing 4 ml of 9000 *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 a final volume of 10 ml with distilled water and incubated for 2 hr at 37°.

At the end of the incubation period, 1 ml of 30% trichloroacetic acid and 1 ml of 1 N H_2SO_4 were added to the reaction mixture and the mixture was centrifuged to remove protein. The supernatant was poured into a distillation flask and formaldehyde was distilled immediately into a graduated tube. An aliquot was taken from the distillate and the amount of formaldehyde was determined by the chromotropic acid method.[7]

Incorporation of ^{14}C-Labeled Amino Acids——^{14}C-Amino acids (2 μCi) was administered intraperitoneally 2 hr prior to sacrifice. Rats were sacrificed by decapitation and the liver was removed rapidly into cold 0.25 M sucrose solution, weighed and homogenized in 9 volumes of Medium A[8] (0.25 M sucrose, 0.025 M $MgCl_2$, and 0.05 M Tris buffer of pH 7.4) in a Potter-Elvehjem type homogenizer in an ice bath.

A nuclei and cell debris were removed by centrifugation at 700 *g* for 10 min. A mitochondrial fraction was sedimented from 700 *g* supernatant at 8000 *g* for 10 min. A microsomal fraction was sedimented by centrifugation of the 8000 *g* supernatant at 105000 *g* for 60 min. A soluble fraction was precipitated from 105000 *g* supernatant by addition of cold 6 N $HClO_4$. Each fraction, except the nucleus and cell debris fraction, was washed three times with cold 0.5 N $HClO_4$, and treated with EtOH-ether-$CHCl_3$ (2:2:1) mixture to remove lipids. Each sample was treated with 1 N NaOH, kept for 1 hr at room temperature, again precipitated by 6 N HCl, and washed three times with 0.5 N $HClO_4$. The nucleus and cell debris fraction was washed three times with cold 0.5 N $HClO_4$ at 70° for 15 min to remove deoxyribonucleic acid and treated as described above. An aliquot of each sample fraction was dissolved in 3 ml of 10% NH_4OH. One ml of each sample was placed on a planchet, dried under a heat lamp, and the radioactivity was counted by a gas-flow counter. The protein content of each sample was determined by the Biuret method.[9]

Results

I. Effect of Adrenalectomy and Cortisone on Oxidative Demethylation

As shown in Fig. 1, oxidative demethylation of N-methyl compounds was markedly decreased by adrenalectomy. In adrenalectomized rats, the activity of oxidative demethylation was not restored to the control level by the administration of 5 mg/kg of cortisone for 3 days, but was considerably recovered by the administration of 25 mg/kg of cortisone for 3 days.

II. Time Course of Stimulative Effect of Phenobarbital in the Adrenalectomized Rats

As shown in Fig. 2, stimulating effect of phenobarbital on the demethylating activities appeared in the adrenalectomized rats as well as in intact animals.[6] The maximum stimulation was obtained around 40—50 hr after pretreatment with phenobarbital. A similar time course of the activities was observed when meperidine was used as a substrate instead of methylbarbital, but demethylation of morphine was not activated under the same conditions.

III. Effect of Cortisone and Phenobarbital on the Oxidative Demethylation in Adrenalectomized Rats

Effect of phenobarbital and cortisone on the oxidative demethylation in the liver of adrenalectomized rat is shown in Fig. 3. Synergistical activation of demethylation could not be

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7) R.M. Burton, *Methods in Enzymology*, **3**, 247 (1957).
8) J.W. Littlefield and E.B. Keller, *J. Biol. Chem.*, **224**, 13 (1959).
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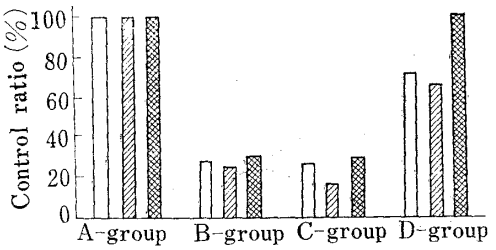

Fig. 1. Effect of Adrenalectomy and Cortisone on the Oxidative Demethylation

A: intact control
B: adrenalectomy
C: adrenalectomy+cortisone (5 mg/kg)
D: adrenalectomy+cortisone (25 mg/kg)
□ meperidine ▨ methylbarbital
▨ morphine

$$\text{control ratio} = \frac{\text{activity after pretreatment}}{\text{activity of control}} \times 100\%$$

control values: meperidine 2.35±0.3 μmoles
morphine 1.00±0.2
methylbarbital 0.35±0.1

Values are expressed as mean ± standard deviation.

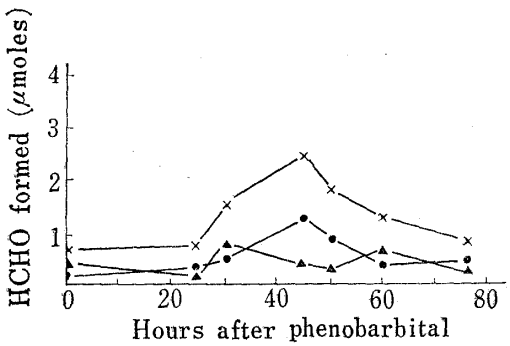

Fig. 2. Time Course of Oxidative Demethylation in the Liver from Adrenalectomized Rats after Administration of Phenobarbital

substrate
—×—×— meperidine
—●—●— methylbarbital
—▲—▲— morphine

recognized at a lower dose of cortisone against a single administration of phenobarbital, whereas a higher dose, 25 mg/kg of cortisone showed a marked activation of demethylation over the level elevated by treatment with phenobarbital.

The activity of demethylation of morphine was not affected by a single treatment with phenobarbital, but was increased by the effect of both phenobarbital and cortisone. Therefore, it is conceivable that demethylation of morphine is conducted by enzyme systems different from the demethylation of meperidine or methylbarbital against the activation of phenobarbital.

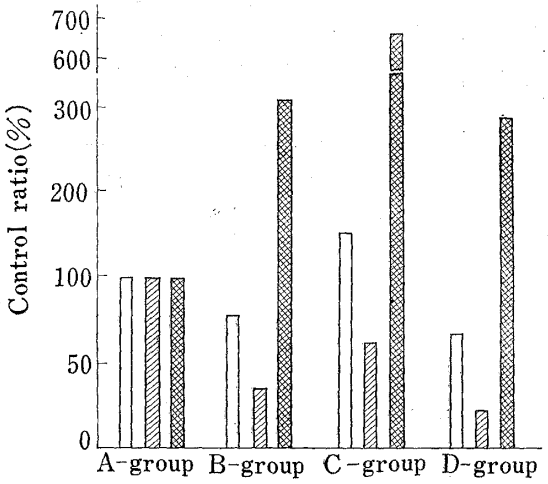

Fig. 3. Effects of Cortisone and Phenobarbital on the Oxidative Demethylation in the Liver from Adrenalectomized Rats

A: intact control
B: adrenalectomy+cortisone (5 mg/kg)+phenobarbital
C: adrenalectomy+cortisone (25 mg/kg)+phenobarbital
D: adrenalectomy+phenobarbital
Control ratio and control values are as described in Fig. 1.
□ meperidine ▨ morphine ▩ methylbarbital

IV. Effect of Cortisone in Intact Animals

In order to find the effect of cortisone on intact and starved rats, alteration in the activities of oxidative demethylation after the administration of cortisone was investigated.

As shown in Fig. 4, a and b, the activity of oxidative demethylation was slightly stimulated for 10 hr by the administration of 5 mg/kg of cortisone and the maximum peak appeared around 3—7 hr. This effect decreases after 10 hr in the rat starved for 48 hr.

V. Incorporation of ^{14}C-Labeled Amino Acids *in Vivo* into the Liver Protein of Adrenalectomized Rats

The relationship between protein synthesis and activity of demethylating enzyme was examined. The incorporation of ^{14}C-labeled amino acids into the subcellular fraction of liver in the intact animals is shown in Fig. 5.

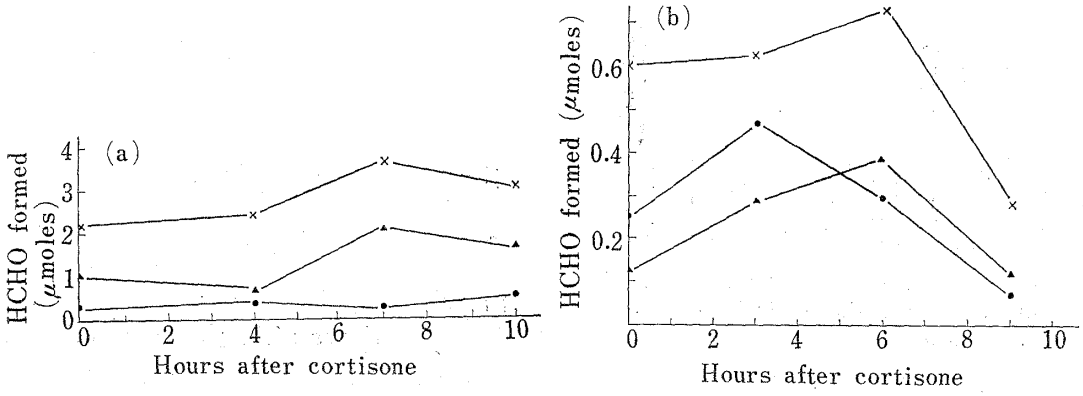

Fig. 4. Effect of Cortisone on Oxidative Demethylation

a) intact rats

b) starved rats
Rats were starved for 48 hours, intraperitoneally administered in a dose of 5 mg of cortisone/kg and killed 0, 3, 6, and 9 hours after the administration of the drug.

—×—×— meperiidine —▲—▲— morphine —●—●— methylbarbital

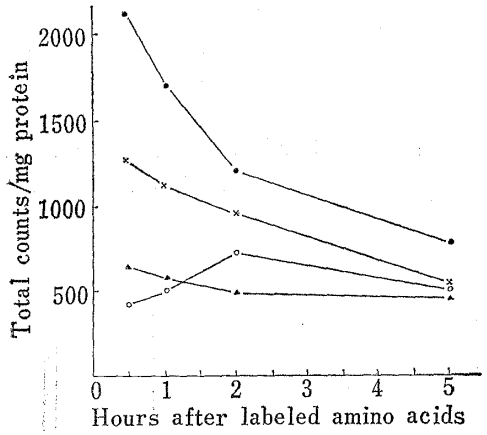

Fig. 5. Time Course of the Incorporation of ^{14}C-labeled Amino Acids in Intact Animals

Rats were intraperitoneally administered ^{14}C-labeled amino acids (2 μCi) and sacrificed 0.5, 1, 2, and 5 hours later.
—●—●— microsomal fraction
—×—×— mitochondrial fraction
—▲—▲— nucleous and cell debris
—○—○— soluble fraction

The rate of incorporation of ^{14}C-labeled amino acids was compared 2 hr after the administration of ^{14}C-amino acids. Fig. 6, (a) and (b), shows that the incorporation of ^{14}C-labeled amino acids into microsomal protein was increased more significantly than other fractions by the administration of phenobarbital, and that the pattern of incorporation of amino acids was similar in both intact and adrenalectomized rats. Effect of phenobarbital on the incorporation rate disappeared after 100 hr.

VI. Inhibitory Effect of Puromycin and 8-Azaguanine on the Stimulation of Phenobarbital

Effect of puromycin and 8-azaguanine on phenobarbital-induced increase in the oxidative demethylation is shown in Table I. The results of these studies reveal that they block the effect of phenobarbital.

Discussion

Numerous studies have demonstrated that drug response accompanies physiological and artificial stimuli in higher animals. Among these, it is especially pertinent to note the work of Remmer[10] who suggested that hormones may act as a direct stimulant. Remmer reported[11] that adrenalectomy lowered the activity of liver microsomal enzymes that N-demethylated monomethyl-4-aminoantipyrine and that oxidize hexobarbital, and administration of predonisolone or cortisone to intact rats resulted in a shortened duration of hexobarbital action and an accelerated metabolism of barbiturates by the liver.

In our investigation, effects of inductive drugs, hormone, and inhibitors were studies in intact and adrenalectomized rats.

The results showed that the activities of demethylating enzymes are markedly decreased in the liver microsomes by adrenalectomy. The lowered activity caused by adrenalectomy

10) H. Remmer, *Naturwissenshaften*, **45**, 522 (1958).
11) H. Remmer, *Arch. Exptl. Pathol. Pharmakol.*, **223**, 184 (1958).

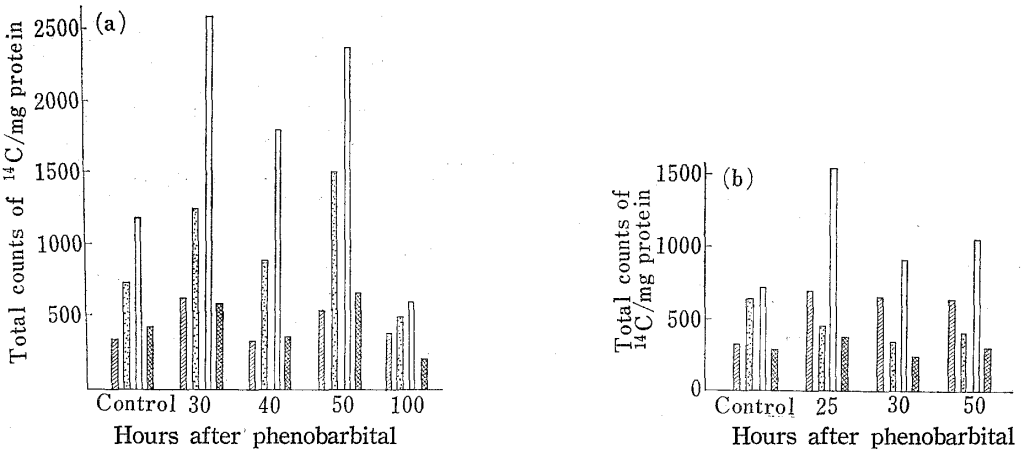

Fig. 6. Effect of Phenobarbital on the Incorporation of ^{14}C-labeled Amino Acids into Liver Proetins *in Vivo*

a) intact rats
Rats were administered orally 500 μmoles/kg of phenobarbital and were killled 30, 40, 50, and 100 hours after administration of the drug.

b) adrenalectomized rats
Rats were orally administered 500 μmoles/kg of phenobarbital and were killed 25, 30, and 50 hours after the administration of the drug.

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

TABLE I. Effect of Puromycin and 8-Azaguanine on the Stimulation of Phenobarbital

Pretreatment	Substrate	No. of animals	Demethylation activity[a]	Ratio to control	Inhibition %
None	methyl barbital	6	0.26±0.09	1	
	meperidine	6	1.50±0.34	1	
	morphine	6	0.61±0.19	1	
Phenobarbital	methyl barbital	5	1.61±0.25	6.19	
	meperidine	5	2.44±0.45	1.63	
	morphine	5	0.80±0.30	1.31	
Phenobarbital and puromycin	methyl barbital	3	0.29±0.11	1.12	81.8[b]
	meperidine	3	1.29±0.28	0.86	47.2
	morphine	3	0.68±0.13	1.11	15.3
8-Azaguanine	methyl barbital	3	0.14±0.09	0.54	46[c]
	meperidine	3	0.93±0.22	0.62	38
	morphine	4	0.73±0.17	1.20	
Phenobarbital and 8-azaguanine	methyl barbital	3	0.48±0.07	1.85	70.2[b]
	meperidine	3	1.82±0.19	1.20	25.4
	morphine	4	0.56±0.08	0.92	30

a) formaldehyde formed μmoles/g liver/2 hours
Values are expressed as mean±standard deviation.

b) inhibition (%) $=100-\frac{A}{B}\times 100$

A: activity after pretreatment with phenobarbital and inhibitor
B: activity after pretreatment with phenobarbital

c) inhibition (%) $=100-\frac{\text{pretreated activity with inhibitor}}{\text{control activity}}\times 100$

Rats were orally administered 500 μmoles/kg of phenobarbital 24 hours prior to sacrifice.

Puromycin, 10 mg/kg, was intraperitoneally injected to the rat four times every 2 hours and rats were sacrificed 3 hours after the last injection.

Rats were injected with 100 mg/kg of 8-azaguanine intraperitoneally three times every 15 hours and sacrificed at 9 hours after the last injection.

is recovered completely by pretreatment with a high dose of cortisone, even though in case of intact animals, demethylating activities are slightly increased. Therefore, it can be considered that cortisone relates with these enzymes. It appeared that the effects of phenobarbital and cortisone on N–demethylating enzyme in adrenalectomized rats were additive and, as was previously found, that the plasma level of corticosterone[12] is not significantly changed after pretreatment with phenobarbital in intact rats. Therefore, it is likely that the phenobarbital effect is not mediated through adrenal hormones.

From the data of our experiments, the activities of demethylating enzymes were not affected by pretreatment with low dosage of cortisone, 5 mg/kg doses daily for three days, 24 hr before sacrifice but, as shown in Fig. 4a and b, it was found that effect of low dose of cortisone appeared within 10 hr and this effect diminished completely 24 hours after pretreatment with the same dose of cortisone.

In general, the factors directly affecting the drug–metabolizing enzymes might play an important role in the biosynthesis of enzyme proteins.

On the other hand, there are some conceptions[13] that activation of drug–metabolizing enzymes may be brought about by substances which activate the penetration of the drug into the site at which drug–metabolizing enzymes occur, such as the liver microsomes.

Koike, *et al.* [14] showed that the incorporation of ^{14}C–alanine and ^{14}C–amino acids into the protein synthesized *de novo* by polyribosome system was increased by the administration of cortisone in intact and adrenalectomized mice. Kato, *et al.*[15] also reported that the protein from the phenobarbital–treated ribosomes was 24% and 35% more radioactive than the comparable control protein using ^{14}C–leucine.

In our experiments, incorporation of radioactive amino acids into liver from the phenobarbital–treated rats was greater than that in the control. Since the same phenomena could be observed in the liver from adrenalectomized rats, it may be considered that phenobarbital does not exert its effect *via* the adrenal. The incorporation of ^{14}C–amino acids into microsomes was higher than those into other subcellular fractions of liver from the rats pretreated with phenobarbital.

Puromycin and 8–azaguanine completely blocked the effect of phenobarbital to increase the activities of enzyme systems in liver microsomes, so that it is likely that the elevation of demethylating enzyme activities by pretreatment with phenobarbital depends on the synthesis of enzyme protein.

With respect to the activation of microsomal enzymes, a possible mechanism would be that such an activation is brought about through the biosynthesis of enzyme protein by the activation of phenobarbital or cortisone as shown in the present experiments. However, the effect of permeability change still remains as a cause of activation.

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14) K. Koike, T. Otaka, and S. Okui, *J. Biochem.*, **61**, 679 (1967).
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