it was stable for fourty minutes, nothing was shown on the photo- and temperature-sensitivity, the presence of some kinds of active methylene compound and reducing substance in reasonable amounts affected negligibly on the absorbances as is shown in Table I, and that 0 to 200 μg./ml. of creatinine were able to be determined with accuracy as is shown in Fig. 1 and Table Ⅲ. When different amounts of creatinine were added to the urine samples, the recovery of added material has been ranged from 99.9% to 103.6% as is shown in Table Ⅱ. An inspection of Table Ⅳ indicated that the results given by the present method was in good agreement that by the Folin-Wu's method.

It seemed most reasonable to conclude that the standard procedure presented in this paper was suitable for the practice of the clinical test.

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Summary

A sensitive and accurate colorimetric determination for urinary creatinine is described. The principle of this method is based on the reaction of creatinine with 2,2,′4,4′-tetranitrobiphenyl in the presence of potassium hydroxide to give a stable color which shows no photo- and temperature-sensitivity and is measured spectrometrically at 555 mμ. The applicable concentration range is 0 to 200 μg./ml. of creatinine. No interferences were shown by considerably larger amounts of acetone, glucose, ascorbic acid, pyruvic acid, and dehydroepiandrosterone.

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176. Toshihiko Ariyoshi and Eigo Takabatake*1 : Biochemical Studies on the Drug Metabolism. III.*2 Inductive Effect of Malonic Acid Derivatives on Cyclobarbital-Metabolizing Enzyme in Rat Liver.*3

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Many drugs are metabolized by NADPH-dependent enzyme system in liver microsomes.[1,2] Activity of this drug-metabolizing enzyme is affected by various kinds of condition : age, sex, strain, species, or neutritional status of animal,[3~6] the administra-

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tion of drugs which produce various pharmacological actions,[7~16] hormones,[4,17~19] and so on.

Previous paper[20] demonstrated that the cyclobarbital*4 (EHB) metabolizing enzyme activity was stimulated by the pretreatment of rats with EHB itself or one of its metabolites, 3-OH-EHB (5-ethyl-5-(3-hydroxycyclohexenyl)barbituric acid), while another metabolite, 3-keto-EHB (5-ethyl-5-(3-oxocyclohexenyl)barbituric acid), did not show any effect in spite of pharmacological inactivities of both metabolites.

This results suggested that the stimulating effect on drug-metabolizing enzyme of various drugs would not depend on their pharmacological action so much.

It is especially interesting that a metabolite which is pharmacologically inactive still has the stimulating action on the drug-metabolizing enzyme. Then, the attention was given to the effect of change in chemical structure on drug-metabolizing enzyme.

The chemical structure of compounds which affect on the drug-metabolizing enzyme is so variable that it is difficult to estimate the relationship between their chemical structure and effect. In this series of experiments, the effect on EHB-metabolizing enzyme activity of malonic acid derivatives having cyclohexene ring and 1-methyl or 1,3-dimethylbarbiturates were examined. The former compounds are considered to be derived by opening the barbituric acid ring of EHB or MHB (Hexobarbital, 5-cyclohexenyl-3,5-dimethylbarbituric acid). The latter compounds are derived by introduction of methyl group into barbituric acid ring.

Experimental

Materials and Methods——Malonic acid derivatives were synthesized from cyclohexanone and ethyl cyanoacetate following the method of Harding, *et al.*,[21] Kon, *et al.*,[22] and Tanaka, *et al.*[23] 2-(Diethylamino)ethyl cyclohexenylethylacetate (X)*5 and N-[2-(diethylamino)ethyl]-2-cyclohexenyl-2-ethylacetamide (XI)*6 were prepared by the same procedure as the preparation of phenylacetic acid derivatives.[24]

The animals used were Wistar female rats and fed "Oriental rat diet-MN" for 1 week prior to the all experiments. Malonic acid derivatives were injected intraperitoneally in single dose as a solution of propylene glycol. Control rats were administered intraperitoneally only with propylene glycol. EHB was injected intraperitoneally as aqueous solution containing 1.1 equiv. of NaOH.

The determination of the duration of EHB-hypnosis and EHB-metabolizing enzyme activity of liver homogenate were performed as previously reported.[20]

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*4 The abbreviation of EHB would be used for 5-ethyl-5-cyclohexenylbarbituric acid in this series although its official name has been changed from ethylhexabital to cyclobarbital in J. P. VII.

*5 X. hydrochloride $C_{16}H_{29}NO_2 \cdot HCl$, m.p. 102~104°. *Anal.* Calcd. C, 63.23; H, 9.91; N, 4.60. Found C, 62.89; H, 9.71; N, 4.84.

*6 XI. oxalate $C_{16}H_{30}N_2O \cdot (COOH)_2$, m.p. 104~106°. *Anal.* Calcd. C, 60.64; H, 9.10; N, 7.85. Found C, 60.04; H, 8.80; N, 8.12.

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Results and Discussion

It is supposed that the compounds shown in Chart 1 are produced by opening the barbituric acid ring of EHB, so the effect on EHB-metabolizing enzyme of these compounds were examined.

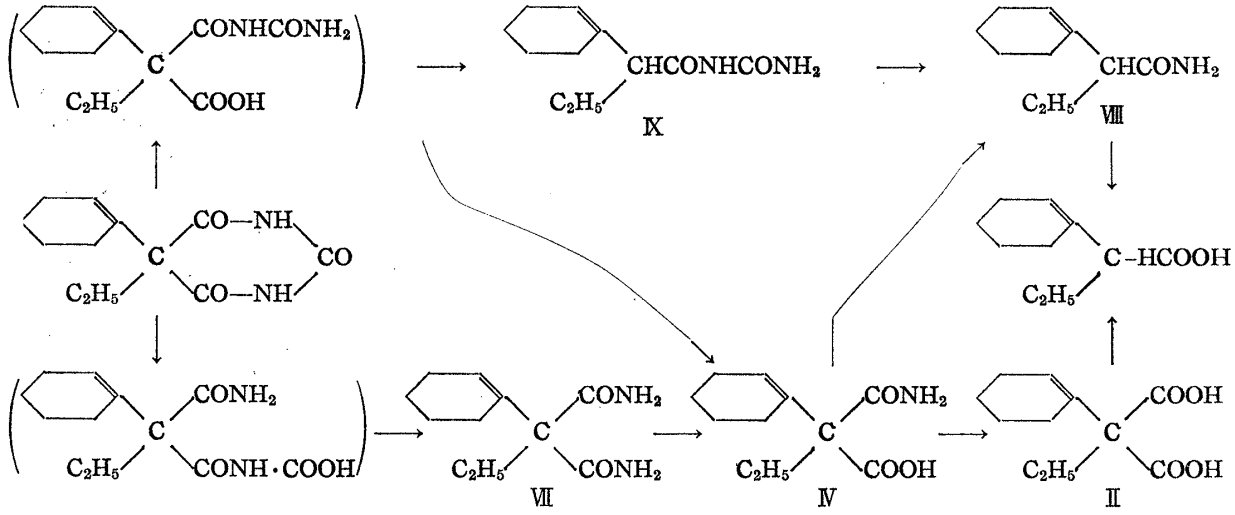
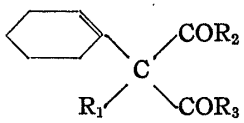
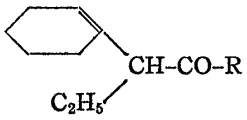


Chart 1. The Compounds are produced by Opening the Barbituric Acid Ring of EHB

TABLE I. The Effect of Malonic Acid Derivatives on the Duration of EHB-Hypnosis



Pretreatment Compd.	R_1	R_2	R_3	No. of rats	Body weight (g.)	Duration of EHB-hypnosis (min.)	*P* value	
Control				11	92	90±5		
I	H	OH	OH	6	89	100±10	>0.05	>0.05
II	C_2H_5	〃	〃	6	86	82±1	>0.05	
III	H	NH_2	〃	6	93	111±8	<0.05	>0.05
IV	C_2H_5	〃	〃	5	95	97±5	>0.05	
V	H	〃	OC_2H_5	8	90	92±5	>0.05	<0.05
VI	C_2H_5	〃	〃	5	94	72±6	<0.05	
VII	〃	〃	NH_2	6	87	42±4	<0.01	



Pretreatment Compd.	R	Dose (mg./kg.)	No. of rats	Body weight (g.)	Duration of EHB-hypnosis (min.)	*P* value
Control			15	94	88±3	
VIII	NH_2	200	5	91	72±3	<0.05
IX	$NHCONH_2$	200	6	84	70±4	<0.05
X	$OCH_2CH_2N(C_2H_5)_2$	100	10	95	124±5	<0.05
			9	99	171±12[a)]	<0.01
XI	$NHCH_2CH_2N(C_2H_5)_2$	100	6	114	116±2	<0.05
			5	98	170±13[a)]	<0.01

Animals received malonic acid derivatives intraperitoneally in single dose (200 mg./kg.) 24 hours before the EHB (80 mg./kg.) injection. except *a*).

a) Rats were treated intraperitoneally in single dose (100 mg./kg.) 1 hour before the EHB (80 mg./kg.) injection. The values present mean±standard error.

The mechanism of inductive activation of drug-metabolizing enzyme is not obvious and it has been known that the one drug showed both stimulating and inhibiting effect depending on the experimental condition. However, in order to compare the influence of minor change in chemical structure of many compounds, a definite condition for pretreatment, single injection 24 hours before EHB administration, was adopted through this experiment with some exception.

Table I shows the effect of malonic acid derivatives on the duration of EHB-hypnosis. The pretreatment of rats with cyclohexenylethylmalonamic acid ethyl ester (Ⅵ), cyclohexenylethylmalonic acid diamide (Ⅶ),2-cyclohexenyl-2-ethylacetamide (Ⅷ), and cyclohexenylethylacetylurea (Ⅸ) shortened the duration of EHB-hypnosis. On the other hand, the pretreatment of rats with cyclohexenylmalonamic acid (Ⅲ) prolonged the duration of EHB-hypnosis. No effect was observed in the pretreatment of cyclohexenylmalonic acid (I), cyclohexenylethylmalonic acid (Ⅱ), cyclohexenylethylmalonamic acid (Ⅳ), and cyclohexenylmalonamic acid ethyl ester (V).

The introduction of ethyl group into R_1 shortened the duration of EHB-hypnosis. While free malonic acid derivatives did not affect the EHB-hypnosis, acid-amide group showed prolonging effect and ethylester group had shortening effect. However, diamide shortened the EHB-hypnosis.

The pretreatment of rats with X or Ⅺ, diethylaminoethyl-derivatives of Ⅷ, prolonged the duration of EHB-hypnosis, especially, the pretreatment 1 hour before EHB-administration showed the greater effect.

Table Ⅱ. The Effect of Malonic Acid Derivatives on EHB-Metabolizing Enzyme Activity

Pretreatment	Dose (mg./kg.)	No. of rats	Body weight (g.)	Remained EHB (%)	Formed 3-OH-EHB (%)	Formed 3-Keto-EHB (%)
Experiment I						
Control		13	115	65.7±1.8	10.8±1.1	4.28±0.50
I	200	5	119	61.6±3.1	18.4±0.7	6.21±1.06
Ⅱ	200	6	120	60.3±1.0	17.2±4.6	3.64±1.67
Ⅲ	200	5	108	70.3±1.4	10.7±0.5	1.76±0.64
Ⅳ	200	5	106	69.6±2.6	6.1±1.0	4.41±0.76
Experiment Ⅱ						
Control		8	108	63.8±1.8	13.6±1.4	2.28±0.90
Ⅵ	200	5	100	46.8±4.5	30.7±2.8	3.83±0.31
Ⅶ	200	8	111	54.0±3.4	15.3±2.0	2.11±0.40
Ⅷ	200	5	118	46.3±6.8	25.5±6.8	7.47±1.41
Ⅸ	200	5	111	57.7±3.8	15.8±1.2	2.45±0.50
Experiment Ⅲ						
Control		15	111	66.1±2.0	14.2±1.6	4.63±0.56
X	100	12	121	74.6±2.9	7.8±2.6	1.77±0.58
X	100×3 days	6	110	81.3±7.4	4.5±2.2	1.10±0.38
Ⅺ	100	4	116	70.1±3.2	14.1±0.2	0.70±0.28

Animals received malonic acid derivatives intraperitoneally in single dose. Twenty-four hours later, animals were sacrificed and EHB-metabolizing enzyme activity of the liver was determined. The values present mean±standard error.

As shown in Table Ⅱ the liver preparation of rats treated with Ⅵ, Ⅶ, Ⅷ, and Ⅸ had higher activities for EHB-metabolism, thus the amount of remained EHB were decreased and the metabolites formed were increased. On the other hand, the treatments with Ⅲ and Ⅳ inhibited the EHB-metabolism. This inhibiting effect was markedly observed in the case of repeated administrations of X.

From these results, it was found that the administrations of Ⅵ, Ⅶ, Ⅷ, and Ⅸ shortened EHB sleeping time by stimulating the EHB-metabolizing enzyme system in the liver and that the treatments with Ⅱ and Ⅹ prolonged EHB sleeping time by inhibiting the EHB-metabolizing enzyme system in the liver. Among these compound, Ⅷ and Ⅸ showed hypnotic action but Ⅱ, Ⅳ, and Ⅶ had no pharmacological action in the doses used. However, Ⅶ and Ⅷ had stimulating effects on EHB-metabolizing enzyme, so the activation of drug-metabolizing enzyme does not seem to relate to the pharmacological activity.

Many drugs which inhibit drug-metabolizing enzyme have diethylaminoethyl group such as Kramer's 5712 (ethylbutylmalonic acid ethyl (2-diethylamino)ethyl diester),[16] SKF-525A (2,2-diphenylvaleric acid 2-(diethylamino)ethyl ester),[12] and so on. No effect of diethylaminoethanol on drug-metabolizing enzyme has been reported by Cooper, *et al.*[25] Ⅷ stimulated the EHB-metabolism, but its diethylaminoethyl ester (Ⅹ) or amide (Ⅺ) inhibited by contraries. So the introduction of diethylaminoethyl group seemed to be considerably important for inhibiting drug-metabolizing enzyme.

From these experiments, it was found that only minor change in chemical structure of the malonic acid derivatives having cyclohexene ring-series could result in a remarkable change in drug-metabolizing enzyme activity.

The authors are indebted to Shionogi Co., Ltd. for their supply of cyclobarbital.

Summary

Malonic acid derivatives having cyclohexene ring were prepared and their effects on the metabolism of EHB in the rat liver preparation were examined.

In the female rats pretreated with cyclohexenylethylmalonamic acid ethyl ester (Ⅵ), cyclohexenylethylmalonic acid diamide (Ⅶ), 2-cyclohexenyl-2-ethylacetamide (Ⅷ), and cyclohexenylethylacetylurea (Ⅸ), the duration of EHB-hypnosis was shortened and the EHB-metabolizing activity of liver homogenate was stimulated. On the other hand, the duration of EHB-hypnosis was prolonged and the metabolizing activity was inhibited by pretreatment with 2-(diethylamino)ethyl cyclohexenylethylacetate hydrochloride (Ⅹ) or N-[2-(diethylamino)ethyl]-2-cyclohexenyl-2-ethylacetamide oxalate (Ⅺ).

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