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Studies on Synthetic Sweetening Agents. XV.[1] Metabolism of Sodium Cyclamate. (4). Influences of Phenylbutazone, Phenobarbital, and Tolbutamide on Metabolism of Sodium Cyclamate in Rabbits

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Some workers[3-7] have already reported on the metabolism of sodium cyclamate (CHS-Na), and they showed that cyclohexylamine was excreted in the urine of laboratory animals and human receiving CHS-Na orally. Also, in the previous papers[1,5,8] of this series, the present authors reported that such metabolites other than cyclohexylamine as cyclohexanone, cyclohexanol, and cyclohexylglucuronide were excreted in the urine of rabbit, rat, mouse, and human which had received CHS-Na orally.

Enzyme induction is a well-known mechanism which modifies the metabolism of a drug. It has been reported by many workers[9-12] in recent years that certain drugs can induce enzymes in the liver that accelerate the metabolism of other drugs. Since CHS-Na has been used as one of synthetic sweetening agents for a corrective of medicinal agents, it is presumed that the metabolism of CHS-Na may be modified by the drugs which are given concomitantly. However, the influences of other drugs on the metabolism of CHS-Na have not so far been reported.

In the present paper, the fates of CHS-Na and its metabolites *in vivo* were investigated in rabbits, and then, the influences of typical enzyme-inducing agents such as phenylbutazone, phenobarbital and tolbutamide on the metabolism of CHS-Na were studied *in vivo* and *in vitro* in rabbits. In addition, the effects of tolbutamide on the metabolism of cyclohexylamine, cyclohexanone, and cyclohexanol were also investigated.

Experimental

Materials——Pure sample of CHS-Na was obtained by repeated recrystallization of reagent grade one, and dried *in vacuo* for 6 hr. Cyclohexylamine, cyclohexanol, cyclohexanone, isoamylacetate, and *n*-butylether were purified by distillation of commercial products of reagent grade. Phenylbutazone, phenobarbital, tolbutamide, and other chemicals used were of reagent grade.

Test Animal, Administration of Drugs, and Collections of Urine——Male rabbits weighing 2.7—3.6 kg were kept on the solid food and water in individual cages. The drugs dissolved or suspended in 50 ml of water were given by a stomach tube and urine was collected in a flask containing toluene for preventing putrefaction.

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Apparatus and Experimental Conditions of Gas Liquid Chromatography——A Shimadzu Model GC-3AF gas chromatograph was used. Nitrogen was used as a carrier gas. The column (300 cm × 3 mm *i.d.*, stainless steel coil-tube) contained a packing with 20% PEG 20M and 2.5% NaOH on 60—80 mesh Shimalite.

The experimental conditions were as follows: The column temperatures were maintained at 130° for cyclohexylamine and at 140° for cyclohexanone and cyclohexanol. The flow rates of carrier gas for cyclohexylamine, cyclohexanone, and cyclohexanol were 30 ml/min, 22.5 ml/min, and 22.5 ml/min, respectively.

Determination of Unchanged CHS-Na in Urine——Each of samples for the preparation of a calibration curve was prepared by an addition of any dose between 0.4 and 3.0 mg of pure CHS-Na to 1 ml of rabbit urine. To 1 ml of sample solution were added 1 ml of water, 0.5 ml of 3N HCl and 0.5 ml of 2% H_2O_2. The mixture was heated at 100° for 1 hr, basified by adding 1 ml of 10N NaOH, and extracted with 4 and 2 ml of $CHCl_3$ successively. The $CHCl_3$ extract was shaken after the addition of 0.2 ml of 2N HCl and evaporated to dryness. The residue was dissolved in 0.05—5.0 ml of 30 mg%(w/v) isoamylacetate–$CHCl_3$ solution, and shaken with a small amount of anhyd. K_2CO_3. Two μl of the solution was injected into the gas chromatograph at a fixed sensitivity and range. The peak areas of isoamylacetate (as an internal standard) and cyclohexylamine, which was produced by hydrolysis of CHS-Na, were measured by triangulation. The calibration curve of CHS-Na was obtained by plotting the concentration of CHS-Na against the ratio of peak area of cyclohexylamine to that of isoamylacetate.

The amount of unchanged CHS-Na in the urine of rabbit was determined by the procedures described above.

Determinations of Cyclohexylamine, Cyclohexanone, Cyclohexanol, and Cyclohexylglucuronide in Urine——The gas chromatographic determinations of cyclohexylamine, cyclohexanone, cyclohexanol and its glucuronide excreted in the rabbit urine were carried out according to the method for human urine reported in the previous paper.[1]

***In Vitro* Experiment using Rabbit Liver Homogenate**——The rabbits were sacrificed by bleeding. The liver was removed immediately, and washed with ice-cold 0.14M potassium chloride solution. The organ was homogenized in 2 volumes of ice-cold 0.2M phosphate buffer solution (pH 7.5) with a teflon pestle glass homogenizer and 0.5 ml of 0.1M nicotinamide solution per 1 g tissue was added. The homogenate was divided into four equal portions, 100 mg of CHS-Na, 10 mg of cyclohexylamine, 10 mg of cyclohexanone, and 10 mg of cyclohexanol were added to each portion respectively, and incubated aerobically for 2 hr (shaking rate, 120/min, at 37°). The supernatant was obtained by centrifugation at 9000 × *g* for 20 min. The metabolites such as cyclohexylamine, cyclohexanone, and cyclohexanol in the supernatant were determined according to the method reported in the previous paper.[1]

Result and Discussion

Metabolism of Cyclohexylamine, Cyclohexanone, and Cyclohexanol

In order to clarify the metabolic fate of CHS-Na, a single dose of cyclohexylamine (100 mg/animal), cyclohexanone (50 mg/animal), or cyclohexanol (50mg/animal) was administered orally to rabbits. The amounts of unchanged substances and metabolites in urine were shown in Table I. In the rabbits receiving cyclohexylamine, the amount of unchanged cyclohexylamine in the 24 hours-urine was about 20% of the initial dose, and cyclohexanone, cyclohexanol and its glucuronide were found as the metabolites of cyclohexylamine in the urine. About 25% of the cyclohexanol was excreted in unchanged state and a small amount of it was found as glucuronide. On the other hand, in case of cyclohexanone, the amount of unchanged substance was extremely small and those of its metabolites such as cyclohexanol and cyclohexylglucuronide were about 18% of the initial dose.

Moreover, the metabolism of cyclohexylamine, cyclohexanone, and cyclohexanol was investigated *in vitro* by using rabbit liver homogenate according to the procedures mentioned above. As shown in Table II, the amount of unchanged cyclohexanone was only about 9% of the original dose and less than that of unchanged substance in the case of cyclohexylamine or cyclohexanol. And in cases of cyclohexanone and cyclohexanol, the total amounts of recovered substances, that is, unchanged substance and metabolites were only about 30% of the initial dose. Also, it has been reported by Elliott, *et al.*[13] that when cyclohexane was administered to the rabbit, cyclohexanol and its glucuronide were the urinary metabolites and that

13) T.H. Elliott, D.V. Parke and R.T. Williams, *Biochem. J.*, **72**, 193 (1959).

TABLE I. Effects of Tolbutamide on the Metabolism of Cyclohexylamine, Cyclohexanone, and Cyclohexanol in Rabbits

Drug	Condition	Days after administration	% excreted[a]				
			Cyclohexylamine	Cyclohexanone	Cyclohexanol	Conjugated cyclohexanol	Total metabolites[b]
Cyclohexylamine	alone	1	19.400[c]	0.230	0.900	0.132	1.262
		2	0.630[c]	0.010	0.074	0.097	0.181
		3	0.235[c]	0	0.010	0.133	0.143
	with tolbutamide	1	2.000[c]	0.075	1.160	0.180	1.415
		2	0.067[c]	0	0.036	0.143	0.179
		3	0.060[c]	0	0.014	0.125	0.139
Cyclohexanone	alone	1	—	0.116[c]	17.760	0.250	18.010
		2	—	0[c]	0.236	0.278	0.510
	with tolbutamide	1	—	0.088[c]	12.200	0.280	12.480
		2	—	0[c]	0.056	0.192	0.248
Cyclohexanol	alone	1	—	0	25.000[c]	0.346	0.346
		2	—	0	0.090[c]	0.222	0.222
	with tolbutamide	1	—	0.010	23.270[c]	0.306	0.316
		2	—	0	0.056[c]	0.218	0.218

a) Each value represents the mean of two experiments. The percent of each metabolite is given in terms of cyclohexylamine, cyclohexanone, or cyclohexanol equivalent.
b) not include unchanged substance
c) percent of unchanged substance
dose: cyclohexylamine (100 mg/animal), cyclohexanone (50 mg/animal), cyclohexanol (50 mg/animal), tolbutamide (120 mg/kg)

TABLE II. Metabolism of Cyclohexylamine, Cyclohexanone, and Cyclohexanol in the Liver Homogenate of Rabbit Pretreated with Tolbutamide (120 mg/kg/day) for 7 Days

Drug	Condition	Substrate added (mg)	% produced[a]			
			Cyclohexylamine	Cyclohexanone	Cyclohexanol	Total metabolites[b]
Cyclohexylamine	alone	7.3	98.800[c]	0.274	0.329	0.603
	pretreatment	7.3	73.500[c]	3.272	5.475	9.747
Cyclohexanone	alone	10.0	—	9.100[c]	24.210	24.210
	pretreatment	10.0	—	1.960[c]	31.020	31.020
Cyclohexanol	alone	10.0	—	6.700	28.100[c]	6.700
	pretreatment	10.0	—	1.600	27.200[c]	1.600

a) Each value represents the mean of two experiments. The percent of each metabolite is given in terms of cyclohexylamine, cyclohexanone, or cyclohexanol equivalent.
b) not include unchanged substance
c) percent of unchanged substnance

carbon dioxide was found in the expired air, although there has been no evidence to demonstrate how carbon dioxide was produced or which metabolite was responsible for the cleavage of a cyclohexane ring.

From the above results, the metabolism of cyclohexanone seems to occur *via* two pathways, one of which is a reducing reaction that changes cyclohexanone to cyclohexanol and another an oxidative one which opens a cyclohexane ring. Thus it is presumed that CHS-Na is primarily metabolized to cyclohexylamine which is subsequently oxidized to cyclohexanone and that cyclohexanone thus produced is further metabolized to cyclohexanol and its glucuronide, and accompanied by the cleavage of cyclohexane ring.

Influences of Phenylbutazone, Phenobarbital, and Tolbutamide on Metabolism of CHS-Na

Rabbit urine was collected for three successive 24 hours periods following the administration of a single dose of 200 mg/kg of CHS-Na with or without 100 mg/kg of phenylbutazone.

The similar investigation was carried out in the dose of 20 mg/kg of phenobarbital or 120 mg/kg of tolbutamide. The amounts of CHS-Na metabolites in urine were shown in Tables III, IV and V, and in Fig. 1. Phenylbutazone and phenobarbital caused an increase in the excretion of CHS-Na metabolites, that is, cyclohexylamine, cyclohexanone, cyclohexanol and its glucuronide. However, tolbutamide caused an increase in the amount of cyclohexylglucuronide alone as a metabolite of CHS-Na.

Also, phenylbutazone, phenobarbital, or tolbutamide was administered orally to rabbits in the same dose as described above for five days in expectation of the induction of enzymes that accelerate CHS-Na metabolism. Then, 200 mg/kg of CHS-Na was administered orally to those rabbits, and its metabolites in urine were determined. As shown in Tables III and IV, and in Fig. 1, the amounts of CHS-Na metabolites in urine of rabbits pretreated with phenylbutazone or phenobarbital were significantly less than those in the rabbits given CHS-Na together with phenylbutazone or phenobarbital and almost the same amount as those in the rabbits received CHS-Na alone. These data suggest that a cyclohexane ring may be cleavaged by further oxidation of cyclohexanone produced, consequently an apparent

TABLE III. Effects of Phenylbutazone on the Metabolism of CHS-Na in Rabbits

Condition	Days after administration	% excreted					
		Unchanged CHS-Na	Cyclohexylamien	Cyclohexanone	Cyclohexanol	Conjugated cyclohexanol	Total metabolites
Alone	1	86.6	0.0100	0.0040	0.0089	0.0293	0.0522
	2	—	0.0022	0.0031	0.0035	0.0308	0.0396
	3	—	0	0	0	0.0235	0.0235
With phenylbutazone	1	83.6	0.0037	0.0062	0.0322	0.0501	0.0922
	2	—	0.0035	0.0010	0.0063	0.0531	0.0639
	3	—	0	0	0.0009	0.0339	0.0348
Pretreated	1	—	0.0033	0.0023	0.0096	0.0405	0.0557
with phenylbutazone	2	—	0.0027	0	0.0080	0.0300	0.0407
for 5 days	3	—	0	0	0.0012	0.0260	0.0272

Each value represents the mean of two experiments.
The percent of each metabolite is given in terms of CHS-Na equivalent.
dose: CHS-Na (200 mg per kg body wt.)
phenylbutazone (100 mg per kg body wt.)

TABLE IV. Effects of Phenobarbital on the Metabolism of CHS-Na in Rabbits

Condition	Days after administration	% excreted					
		Unchanged CHS-Na	Cyclohexylamine	Cyclohexanone	Cyclohexanol	Conjugated cyclohexanol	Total metabolites
Alone	1	—	0.0046	0.0044	0.0054	0.0397	0.0541
	2	—	0.0025	0.0023	0.0026	0.0363	0.0437
	3	—	0.0025	0.0020	0.0021	0.0328	0.0394
With phenobarbital	1	—	0.0079	0.0119	0.0091	0.0949	0.1238
	2	—	0.0028	0.0054	0.0031	0.0639	0.0752
	3	—	0.0014	0.0026	0.0023	0.0562	0.0625
Pretreated	1	—	0.0028	0.0011	0.0065	0.0484	0.0588
with phenobarbital	2	—	0.0021	0	0.0023	0.0449	0.0493
for 5 days	3	—	0	0	0.0007	0.0398	0.0405

Each value represents the mean of three experiments.
The percent of each metabolite is given in terms of CHS-Na equivalent.
dose: CHS-Na (200 mg per kg body wt.)
phenobarbital (20 mg per kg body wt.)

TABLE V. Effects of Tolbutamide on the Metabolism of CHS-Na in Rabbits

Condition	Days after administration	% excreted					
		Unchanged CHS-Na	Cyclohexylamine	Cyclohexanone	Cyclohexanol	Conjugated cyclohexanol	Total metabolites
Alone	1	82.5	0.0037	0.0016	0.0152	0.0350	0.0555
	2	—	0.0019	0	0.0012	0.0360	0.0391
	3	—	0	0	0.0012	0.0354	0.0366
With tolbutamide	1	78.7	0.0031	0.0010	0.0080	0.0639	0.0760
	2	—	0.0010	0	0.0023	0.0559	0.0592
	3	—	0	0	0	0.0520	0.0520
Pretreated with tolbutamide for 5 days	1	79.3	0.0030	0.0032	0.0615	0.0470	0.1147
	2	—	0.0025	0	0.0018	0.0436	0.0479
	3	—	0	0	0.0005	0.0410	0.0415

Each value represents the mean of two experiments.
The percent of each metabolite is given in terms of CHS-Na equivalent.
dose: CHS-Na (200 mg per kg body wt.)
tolbutamide (120 mg per kg body wt.)

decrease in the total amounts of CHS-Na metabolites may occur. On the other hand, the amount of cyclohexanol excreted during 24 hours increased in the rabbits pretreated with tolbutamide (see Table V and Fig. 1).

Moreover, an *in vitro* experiment for CHS-Na metabolism was carried out using the liver homogenates of rabbits which had been administered tolbutamide (120 mg/kg/day) orally for 7 days. As seen in Table VI, the amounts of CHS-Na metabolites increased conspicuously in the liver of rabbits pretreated with tolbutamide. Thus, it was found that tolbutamide induced the activity of liver enzymes which were responsible for the metabolism of CHS-Na.

In order to clarify which metabolic pathway of CHS-Na was promoted by tolbutamide, the metabolism of cyclohexylamine, cyclohexanone, and cyclohexanol was investigated in rabbits by oral administration of each substance together with tolbutamide and, furthermore, the same kind of experiment was carried out *in vitro* using the liver of rabbits pretreated with tolbutamide (120 mg/kg/day) for 7 days. The results of quantitative determination of urinary metabolites were shown in Table I. The amounts of both unchanged substances in the urine of rabbits, to which cyclohexylamine and cyclohexanone had been given, significantly decreased when pretreated with tolbutamide as compared with those in control rabbits. And the amounts of cyclohexanone and cyclohexanol as the metabolites of cyclohexylamine increased as compared with those in rabbits not pretreated with tolbut-

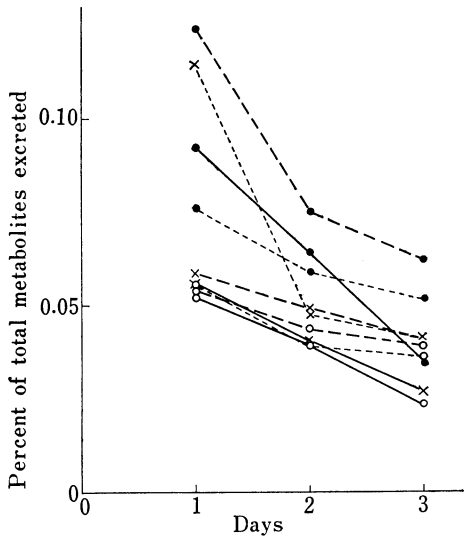

Fig. 1. Effects of Phenylbutazone, Phenobarbital, and Tolbutamide on the Metabolism of CHS-Na in Rabbits

○—○ ○- - -○ ○·····○ : alone
●—●: with phenylbutazone
●- - -●: with phenobarbital
●·····●: with tolbutamide
×—×: pretreated with phenylbutazone for 5 days
×- - -×: pretreated with phenobarbital for 5 days
×·····×: pretreated with tolbutamide for 5 days

TABLE VI. Effects of Tolbutamide on the Metabolism of CHS-Na in Rabbit Liver *in Vitro*

Condition	% produced			
	Cyclohexylamine	Cyclohexanone	Cyclohexanol	Total metabolites
Alone	0.0094	0.0072	0.0038	0.0204
Pretreated with tolbutamide for 7 days	0.6020	0.0422	0.0764	0.7206

Each value represents the mean of two experiments.
The percent of each metabolite is given in terms of CHS-Na equivalent.
the amount of substrate: CHS-Na (100 mg)
the dose of tolbutamide: 120 mg per kg body wt. daily

amide, whereas almost no change occurred in the metabolism of cyclohexanol when pretreated with tolbutamide. Also, as shown in Table II, the influences of tolbutamide on the metabolism of cyclohexylamine, cyclohexanone, and cyclohexanol in rabbit liver homogenate were almost the same as those in the *in vivo* experiment described above. These results suggest that tolbutamide highly intensifies the activity of liver enzymes which oxidize cyclohexylamine to such an extent that the cleavage of cyclohexane ring follows.

Accordingly, it may be concluded that the metabolism of CHS-Na is accelerated by phenylbutazone, phenobarbital, and tolbutamide in the rabbit.

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Effect of Diphenylhydantoin on the Drug Metabolism and the Fatty Acid Composition of Phospholipids in Hepatic Microsomes[1]

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Drug metabolizing enzymes of hepatic microsomes are induced by various chemicals which are roughly classified into two types, a representative of one group is phenobarbital (PB) and another is 3-methylcholanthrene (MC).[3] There are some differences between these two types in the activation of drug metabolizing enzymes and in the influence on microsomal cytochromes.

We found that ethanol and/or ethionine enhanced apparent aniline hydroxylase activity and pointed out the difference between PB and ethanol and/or ethionine in the effects on microsomal phospholipids.[4,5]

1) This work was presented at the 91st Annual Meeting of Pharmaceutical Society of Japan, Fukuoka, April 1971.
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