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Studies on Synthetic Sweetening Agents. XVI.[1] Metabolism of Sodium Cyclamate. (5). The Metabolism of Sodium Cyclamate in Rabbits and Rats after Prolonged Adiministration of Sodium Cyclamate

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1. Urinary excretion of CHS-Na metabolites increased in rabbits and rats after prolonged administration of CHS-Na.

2. The metabolism of CHS-Na was investigated in rabbits pretreated with cyclohexylamine, cyclohexanone, or cyclohexanol and the urinary excretion of CHS-Na metabolites increased in the animals pretreated with cyclohexylamine or cyclohexanone.

3. The produced amounts of CHS-Na metabolites increased in the liver of CHS-Na-treated rabbit and scarcely changed in the liver of CHS-Na-treated rat.

4. The metabolism of cyclohexylamine showed an increase of urinary excretion of cyclohexylamine metabolites in rabbit and rat following prolonged administration of cyclohexylamine. On the other hand, the *in vitro* metabolism of cyclohexylamine resulted in an increase of cyclohexylamine metabolites in the liver of the cyclohexylamine-treated rabbit and did not show a conspicuous difference between the livers of control and the cyclohexylamine-treated rats.

5. These results demonstrated that prolonged administration of CHS-Na caused a stimulation of rabbit liver enzymes metabolizing CHS-Na and potentiated a certain process degrating CHS-Na in rat gut.

Sodium cyclamate (CHS-Na) had been widely used as one of noncaloric sweetening agents in soft drinks and artificially sweetened foods until the agent was recently removed from the market. Some investigators[3–5] have reported that CHS-Na was nonmetabolizable and excreted largely unchanged in both laboratory animals and human receiving CHS-Na. However, since the authors[6] have reported that cyclohexylamine, as a metabolite of CHS-Na, is excreted in the urine of human and dog receiving CHS-Na, many investigations have shown

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3) L.F. Audrieth and M. Sveda, *J. Org. Chem.*, **9**, 89 (1944).
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that CHS-Na can be converted to cyclohexylamine by humans[7-10] and animals.[11-14]

Wallace and his co-workers[14] have studied on the metabolism of sodium and calcium cyclamates in rats after prolonged feeding of those cyclamates in the diet for a year or more, and reported that the prolonged or chronic administration of the cyclamates seemed to potentiate the conversion of the agents to cyclohexylamine. Also, the authors have recently found that cyclohexylamine, cyclohexanone, cyclohexanol and its glucuronide were excreted in the urine of rabbits and rats which had continued to receive CHS-Na orally.[11]

In the present paper, the quantitative variation of CHS-Na metabolites, namely cyclohexylamine, cyclohexanone, and cyclohexanol, was investigated in the urine of and liver of rabbits and rats after prolonged administration of CHS-Na. Furthermore, in order to clarify how the quantitative variation of CHS-Na metabolites caused in the rabbit and rat after prolonged administration of CHS-Na, the metabolism of CHS-Na was investigated in rabbits pretreated with cyclohexylamine, cyclohexanone, or cyclohexanol and also an investigation on the metabolism of cyclohexylamine was carried out in both *in vivo* and *in vitro* methods using rabbits and rats.

Experimental

Materials——CHS-Na was recrystallized from aqueous EtOH, and dried at 105° for 2 hr. Cyclohexylamine hydrochloride (mp 204°) was recrystallized from EtOH. Cyclohexanol, cyclohexanone, isoamylacetate, and *n*-butylether were same as in the previous paper.[1] All other materials were of reagent grade.

Test Animals, Dosage, and Collection of Urine——Male and female rabbits weighing about 3 kg were kept on the solid food and 400 ml of water containing CHS-Na (1 g/animal/day) or cyclohexylamine hydrochloride (50 mg/animal/day) in individual metabolic cages. At various times, the rabbits were given 1 g of CHS-Na or 50 mg of cyclohexylamine hydrochloride dissolved in 50 ml of water by stomach tube and the 24 hr urine was collected in the flask containing toluene for preventing putrefaction. The animals did not receive CHS-Na or cyclohexylamine hydrochloride during the collection of 24 hr urine.

Wistar male and female rats weighing 150—250 g were allowed the solid food and 30 ml of water containing CHS-Na (100 mg/animal/day) or cyclohexylamine hydrochloride (5 mg/animal/day) in individual metabolic cages. The rats received 100 mg of CHS-Na or 5 mg of cyclohexylamine hydrochloride dissolved in 4 ml of water using a stomach sonde and the 24 hr urine was collected according to the method mentioned in rabbits.

Apparatus and Conditions of Gas Liquid Chromatography——The apparatus and conditions of gas liquid chromatography were same as in the previous paper.[1]

Determination Method of Unchanged CHS-Na in the Urine——Unchanged CHS-Na, which was excreted in the urine of rabbit and rat receiving CHS-Na, was determined according to the gas chromatographic method reported in the previous paper.[1]

Determination Methods of Cyclohexylamine, Cyclohexanone, Cyclohexanol, and Conjugated Cyclohexanol in the Urine——Cyclohexylamine, cyclohexanone, cyclohexanol, and conjugated cyclohexanol, which were excreted in the urine of rabbit and rat receiving CHS-Na or cyclohexylamine, were determined by the gas chromatographic methods described in the previous paper[8] of this series.

***In Vitro* Experiments using Livers of Rabbit and Rat**——The rabbits were sacrificed by blood letting from the carotid artery and the rats by decapitation after fasted about 24 hr prior to use in experiments. Immediately after sacrificing the animals, the livers were removed and washed with ice-cold 0.14M potassium chloride solution. Each washed liver was homogenized in two 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. Half a volume of one rabbit liver homogenate or the liver homogenate of two rats was used in one experiment. Each liver homogenate containing 100 mg of CHS-Na or 10 mg of cyclo-

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Table I. Metabolism of CHS-Na following Prolonged Administration of CHS-Na in Rabbit (1 g per animal)

Rabbit No.	Metabolites	μg excreted, day											
		1	2	3	7	14	21	28	35	43	50	57	64
1	cyclohexyl-amine	13.2	30.3	68.4	149.8	539.0	729.0	32370	43080	40820	25120	394.6	50.7
	cyclohexa-nol	0	0	0	0	0	63.9	76.8	*a*)	17.7	444.0	54.4	17.7
	cyclohexa-none	0	0	0	0	0	115.8	935.0	30.3	323.0	*a*)	*a*)	0
	% total metabolites[b)]	0.0027	0.0062	0.0139	0.0303	0.1090	0.1716	6.762	8.7662	8.2162	5.11	0.081	0.0103
2	cyclehexyl-amine	1.3	46.7	15.5	14.1	18.4	114.2	71.1	32.9	150.0	46.2	18.4	45.4
	cyclohexa-nol	0	0	0	0	0	0	0	0	0	0	0	0
	cyclohexa-none	0	0	0	0	0	0	0	0	*a*)	*a*)	0	0
	% total metabolites[b)]	0.0003	0.0095	0.0031	0.0029	0.0037	0.0232	0.0144	0.0067	0.0344	0.0094	0.0037	0.0092
3	cyclohexyl-amine	1.3	40.5	151.3	122.2	131.5	933.0	26400	514.0	631.0	572.5	21.0	26.7
	cyclohexa-nol	0	0	0	0	0	0	50.3	0	*a*)	2.7	0	0
	cyclohexa-none	0	0	0	0	0	0	128.5	*a*)	*a*)	27.8	0	*a*)
	% total metabolites[b)]	0.0003	0.0082	0.0307	0.0248	0.0267	0.199	5.3864	0.104	0.128	0.1217	0.0043	0.0054

a) extremely small amount
b) The percent of total metabolites is given in terms of CHS-Na equivalent.
rabbit No. 1 and 2: male
rabbit No. 3: female

hexylamine hydrochloride was incubated aerobically on an incubator at 37° for 3 hr, heated at 100° for about 5 min, and centrifuged at 9000×*g* for 20 min. The supernatant was treated according to the procedure reported in the previous paper[8] in order to determine the metabolites of CHS-Na and cyclohexylamine.

Result and Discussion

In Vivo Metabolism of CHS-Na in Rabbit after Prolonged Administration of the Agent

At various times, 1 g of CHS-Na was administered to rabbits continued to receive 1 g of CHS-Na/animal/day and the metabolites of CHS-Na were determined in the urine which was collected for 24 hours following the oral administration of the agent. As shown in Table I, the urinary amount of cyclohexylamine, which had been only small amount in a single dose of CHS-Na, increased gradually in accordance with a prolongation of period administering CHS-Na resulting in the urinary excretion ranged from 0.02 to 8.6% of the dose.

In order to clarify how the metabolism of CHS-Na was potentiated, CHS-Na metabolism was investigated in the rabbit pretreated with 10 mg/animal of cyclohexylamine, cyclohexanone, or cyclohexanol for 7 days as compared with that in control animal. As can be seen from Tables II and III, the total urinary amounts of CHS-Na metabolites in the first day apparently increased in the rabbits pretreated with cyclohexylamine or cyclohexanone as compared with those in control animals, although they in the second and third day slightly increased in the cyclohexylamine-pretreated rabbits but somewhat decreased in the cyclohexanone-pretreated animals (see Table II and III). On the other hand, the total amounts of CHS-Na metabolites in the urine of rabbits pretreated with cyclohexanol were almost the same with those in control animals (see Table IV). Thus, these data suggest that CHS-Na metabolites may stimulate CHS-Na metabolism, consequently the metabolites of CHS-Na increase in the rabbits after prolonged administration of CHS-Na.

Table II. Metabolism of Sodium Cyclamate in Rabbit pretreated with Cyclohexylamine

Condition	Days	% of metabolites excreted				
		Cyclohexylamine	Cyclohexanone	Cyclohexanol	Conjugated cyclohexanol	Total metabolites
Alone	1	0.0035	0.0026	0.0069	0.0672	0.0802
	2	0.0023	0.0015	0.0019	0.0596	0.0653
	3	0	0	0.0009	0.0552	0.0561
Treated with cyclohexylamine	1	0.0035	0	0.0251	0.0915	0.1201
	2	0.0018	0	0.0025	0.0705	0.0748
	3	0	0	0.0012	0.0673	0.0685

Each value represents the mean of two experiments.
The percent of each metabolite is given in terms of CHS-Na equivalent.
CHS-Na (200 mg/kg) was administered 7 days after prolonged administration of cyclohexylamine (10 mg/animal/day) for a week.

Moreover, for the purpose of examining the accumulation of CHS-Na and its metabolites in the rabbits continued to receive the agent, a single dose of 1 g of CHS-Na was administered to rabbits and the urinary amounts of unchanged CHS-Na and cyclohexylamine were determined daily for 5 days. As shown in Fig. 1, it was found that most part of unchanged CHS-Na was excreted in the first day and that a small amount of cyclohexylamine was excreted during 4 to 5 days. This result illustrates that the slow excretion rate of cyclohexylamine is somewhat related to the increase of cyclohexylamine in the rabbits after prolonged administration of CHS-Na.

Other metabolites of CHS-Na, namely cyclohexanone and cyclohexanol, were appeared in the urine after 20 to 30th day in two of three rabbits but those urinary amounts were very

TABLE III. Metabolism of Sodium Cyclamate in Rabbit pretreated with Cyclohexanone

Condition	Days	% of metabolites excreted				
		Cyclohexyl-amine	Cyclohexa-none	Cyclohexa-nol	Conjugated cyclohexanol	Total metabolites
Alone	1	0.0053	0.0012	0.0072	0.0553	0.0690
	2	0.0049	0.0011	0.0025	0.0444	0.0529
	3	0.0039	0.0022	0.0022	0.0489	0.0572
Treated with cyclohexanone	1	0.0075	0.0019	0.0396	0.0349	0.0839
	2	0.0071	0.0008	0.0025	0.0332	0.0436
	3	0.0040	0.0009	0.0020	0.0296	0.0365

Each value represents the mean of five experiments.
The percent of each metabolite is given in terms of CHS-Na equivalent.
CHS-Na (200 mg/kg) was administered 7 days after prolonged administration of cyclohexanone (10 mg/animal/day) for a week.

TABLE IV. Metabolism of Sodium Cyclamate in Rabbit pretreated with Cyclohexanol

Condition	Days	% of metabolites excreted				
		Cyclohexyl-amine	Cyclohexa-none	Cyclohexa-nol	Conjugated cyclohexanol	Total metabolites
Alone	1	0.0024	0	0.0076	0.0598	0.0698
	2	0.0018	0	0.0012	0.0616	0.0646
	3	0	0	0	0.0646	0.0646
Treated with cyclohexanol	1	0.0029	0.0018	0.0079	0.0554	0.0680
	2	0.0018	0	0.0023	0.0627	0.0668
	3	0	0	0.0012	0.0610	0.0622

Each value represents the mean of two experiments.
The percent of each metabolite is given in terms of CHS-Na equivalent.
CHS-Na (200 mg/kg) was administered 7 days after prolonged administration of cyclohexanol (10 mg/animal/day) for a week.

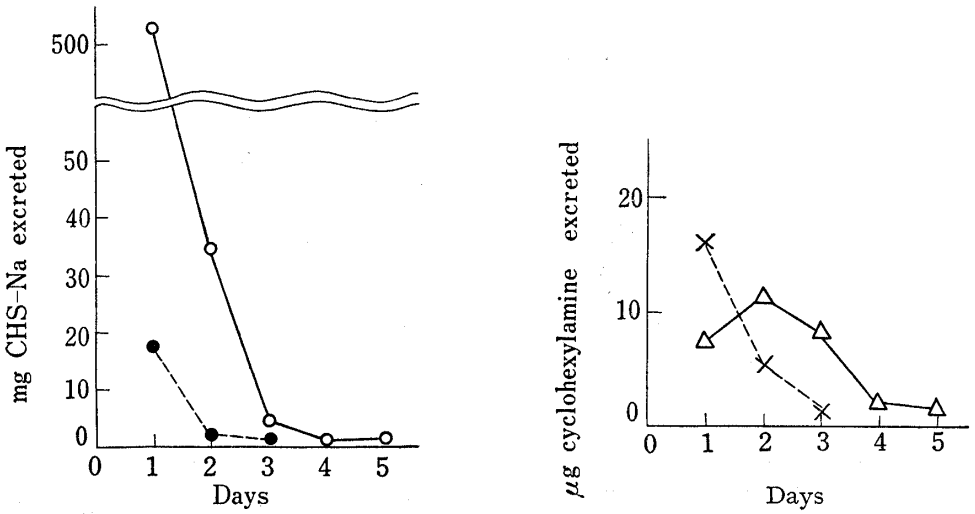

Fig. 1. Unchanged CHS-Na and Cyclohexylamine excreted in the Urine following a Single Oral Dose of CHS-Na

Each plot represents the mean of five experiments.
dose: 1 g of CHS-Na/rabbit, 100 mg of CHS-Na/rat
—○—: unchanged CHS-Na in rabbit ---●---: unchanged CHS-Na in rat
—△—: cyclohexylamine in rabbit ---×---: cyclohexylamine in rat

small compared with that of cyclohexylamine. Furthermore, since 20 to 35 days from the beginning of prolonged administration of CHS-Na, the total urinary amounts of CHS-Na metabolites decreased gradually in accordance with a prolongation of administering period. Also, in the case of prolonged administration of cyclohexylamine to rabbits, the urinary amounts of its metabolites, namely cyclohexanone and cyclohexanol, increased gradually and thereafter had the tendency to decrease with the elapse of administering period (see Fig. 2). As reported in the previous paper,[1] these results suggest that an apparent decrease of total amounts of CHS-Na metabolites occurs on the basis of furhter metabolism of cyclohexanone resulting in cyclohexane ring-opening.

In Vivo Metabolism of CHS-Na in Rat after Prolonged Administration of the Agent

In the same manner as in rabbits, 100 mg of CHS-Na was administered to rats after prolonged administration of the agent and the urinary amounts of its metabolites were determined in the 24 hours urine. As indicated in Table V, the amount of CHS-Na metabolite, namely cyclohexylamine, at first increased and thereafter decreased in accordance with a prolongation of administering period. And cyclohexanone as a metabolite of CHS-Na gradually increased and thereafter decreased with the elapse of administering period. When administered CHS-Na orally to rats, unchanged CHS-Na was almost excreted in the first day, whereas cyclohexylamine was slowly excreted during 3 days (see Fig. 1). Accordingly, it is suggested that the accumulation of cyclohexylamine somewhat reflects on the determination of cyclohexylamine in rats continued to receive CHS-Na.

In rats after prolonged administration of cyclohexylamine, the transition of cyclohexanone excretion was almost the same with that in rabbits, on the other hand, cyclohexanol was

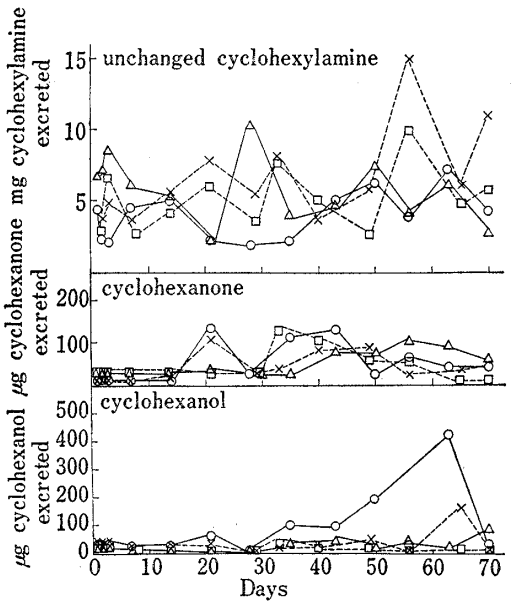

Fig. 2. Urinary Excretion of Cyclohexylamine Metabolites in Rabbit following Prolonged Administration of Cyclohexylamine Hydrochloride (50 mg per animal)

—○— : male rabbit ---□--- : female rabbit

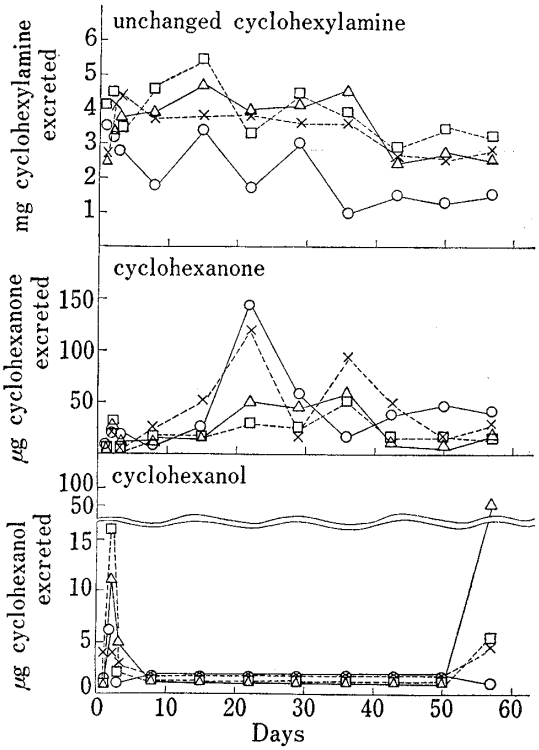

Fig. 3. Urinary Excretion of Cyclohexylamine Metabolites in Rat following Prolonged Administration of Cyclohexylamine Hydrochloride (5 mg per animal)

—○— : male rat ---□--- : female rat

TABLE V. Metabolism of CHS-Na following Prolonged Administration of CHS-Na in Rat (100 mg per animal)

Rat No.	Metabolites	μg excreted, day										
		1	2	3	7	14	21	35	42	49	56	62
1	cyclohexyl-amine	5	8	12	6	6	7	14	7	6	3	1
	cyclohexa-nol	5	3	4	3	13	0	0	0	0	*a*)	0
	cyclohexa-none	8	7	10	8	25	*a*)	77	88	298	60	7
	% total metabolites[b)]	0.0266	0.0306	0.0449	0.0286	0.0630	0.0142	0.1859	0.1957	0.6242	0.1291	0.0164
2	cyclohexyl-amine	*a*)	6	10	22	158	100	107	4	8	8	2
	cyclohexa-nol	20	0	1	2	2	0	0	0	0	*a*)	0
	cyclohexa-none	5	*a*)	5	5	53	20	23	40	93	5	*a*)
	% total metabolites[b)]	0.0103	0.0122	0.0301	0.0549	0.4295	0.2440	0.2643	0.0901	0.2040	0.0265	0.0041
3	cyclohexyl-amine	13	8	9	235	942.5	34	10	7	17.4	18.6	14
	cyclohexa-nol	3	4	1	8	0	0	0	0	*a*)	*a*)	0
	cyclohexa-none	10	7	4	20	20	50	36	205	42	40	10
	% total metabolites[b)]	0.0469	0.0206	0.0265	0.5180	1.9540	0.1716	0.0941	0.4349	0.1216	0.1198	0.0489
4	cyclohexyl-amine	*a*)	9	16	6	312.5	29	91	3.8	9.5	14	1.4
	cyclohexa-nol	*a*)	2	5	6	0	0	0	0	*a*)	23	0
	cyclohexa-none	*a*)	6	12	48	77	28	5	225	45	53	*a*)
	% total metabolites[b)]	*a*)	0.0306	0.0471	0.1107	0.7918	0.1163	0.1849	0.4698	0.1116	0.1371	0.0028

a) extremely small amount
b) The percent of total metabolites is given in terms of CHS-Na equivalent.
rat No. 1 and 2: male
rat No. 3 and 4: female

excreted in an extremely small amount and expected to further convert to its glucuronide (see Fig. 3).

The results obtained above suggest that the conversion ability for CHS-Na rises to a high level in rats following prolonged administration of CHS-Na.

In Vitro Metabolism of CHS-Na in Livers of Rabbit and Rat after Prolonged Administration of the Agent

An *in vitro* metabolism of CHS-Na was investigated using the liver homogenates of rabbits with and without pretreatment with CHS-Na (1 g/animal/day). As shown in Fig. 4, CHS-Na metabolites, namely cyclohexanone and cyclohexanol, significantly increased in the liver homogenates of rabbits pretreated with CHS-Na for 171 and 196 days respectively as compared with those in the animals not pretreated with CHS-Na. On the other hand, the produced amount of cyclohexylamine decreased in the liver of CHS-Na-treated rabbit. This phenomenon illustrates that further metabolism of cyclohexylamine is promoted by prolonged administration of CHS-Na, as would be expected from the data of cyclohexylamine metabolism in the liver homogenate of rabbit pretreated with cyclohexylamine (see Fig. 5). The above results suggest that rabbit liver enzymes metabolizing CHS-Na are stimulated by prolonged administration of the agent.

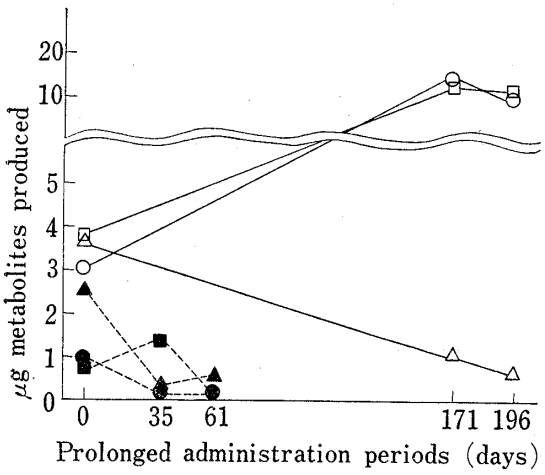

Fig. 4. Metabolism of CHS-Na in the Livers of Rabbit and Rat pretreated with CHS-Na

The amount of substrate: 100 mg of CHS-Na
rabbit —△—: cyclohexylamine
—○—: cyclohexanone
—□—: cyclohexanol
rat ---▲---: cyclohexylamine
---●---: cyclohexanone
---■---: cyclohexanol

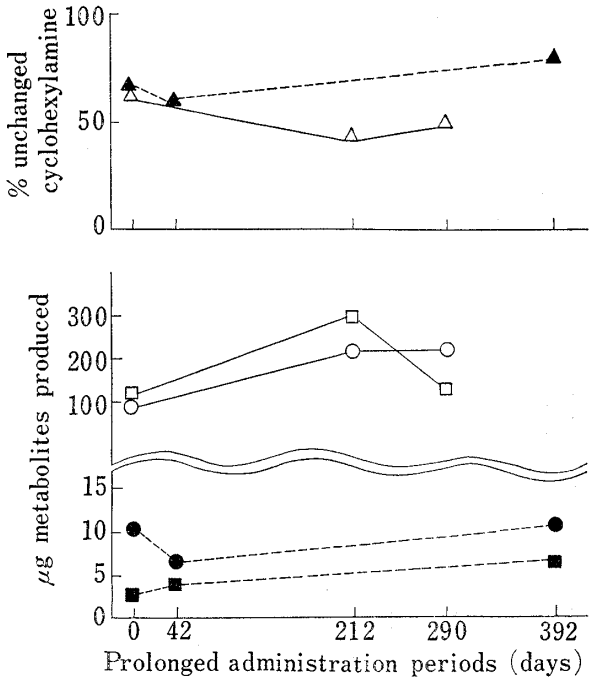

Fig. 5. Metabolism of Cyclohexylamine in the Livers of Rabbit and Rat pretreated with Cyclohexylamine

The amount of substrate: 10 mg of cyclohexylamine hydrochloride
rabbit —△—: unchanged cyclohexylamine
—○—: cyclohexanone
—□—: cyclohexanol
rat ---▲---: unchanged cyclohexylamine
---●---: cyclohexanone
---■---: cyclohexanol

Also, CHS-Na metabolism was investigated in the liver homogenates of rats continued to receive the agent (100 mg/animal/day) for 35 and 61 days respectively. As can be seen from Fig. 4, the produced amounts of CHS-Na metabolites, namely cyclohexylamine, cyclohexanone, and cyclohexanol, were very small and did not show a conspicuous difference

between rats with and without pretreatment with CHS-Na. And in the case of the *in vitro* metabolism of cyclohexylamine in the liver homogenate, the amounts of unchanged cyclohexylamine, cyclohexanone, and cyclohexanol showed only little difference between rats with and without pretreatment with cyclohexylamine as indicated in Fig. 5. It has been shown by some investigations[14–16] that CHS-Na is converted to cyclohexylamine in rat gut. Thus, these data suggest that a metabolizing system in rat liver little participates in the metabolism of CHS-Na and that CHS-Na is predominantly degraded by rat gut-flora.

From both *in vivo* and *in vitro* investigations described above, it is presumed that the prolonged administration of CHS-Na causes a stimulation of rabbit liver enzymes metabolizing the agent and potentiates a certain process degrating the agent in rat gut. Further work is underway and details will be published elsewhere in the near future.

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