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Studies on Synthetic Sweetening Agents. XVIII.[1] Metabolism of Sodium Cyclamate. (7). Dicyclohexylamine, a Metabolite of Sodium Cyclamate in Rabbits and Rats

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Dicyclohexylamine (DCHA), a metabolite of sodium cyclamate (CHS-Na), was identified by gas liquid chromatography (GLC) and thin-layer chromatography (TLC) from the urine of rabbits and rats following repeated oral administration of CHS-Na. The urinary excretions (% of the dose) of DCHA in rabbits and rats receiving CHS-Na were 0.041×10^{-3} and 0.143×10^{-3}%, respectively. Moreover, the metabolic fate of DCHA was investigated in rabbits and rats. The urinary and fecal excretions of unchanged DCHA were relatively small in both rabbits and rats receiving oral or intravenous administration of DCHA. In addition, DCHA was suggested to be primarily metabolized by oxidative reaction in the hepatic $10000 \times g$ supernatant. Thus, it may be due to further metabolism of the produced DCHA that only a small amount of DCHA is excreted in the urine after repeated oral administration of CHS-Na to rabbits and rats.

Keywords——sodium cyclamate; oral administration; dicyclohexylamine; rabbit urine; rat urine; metabolism

Sodium cyclamate (CHS-Na) had been widely used as a noncaloric sweetening agent in soft drinks and artificially sweetened foods until the agent was removed from the market, because it was suspected of being a bladder carcinogen in rats.[2] Kojima and Ichibagase[3] and Leahy *et al.*[4] found cyclohexylamine in the urine of dogs and humans after oral dosing of CHS-Na. Many investigators have already reported that metabolites of CHS-Na, such as cyclohexylamine, cyclohexanone, cyclohexanol and its glucuronide, are excreted in the urine of rabbits and rats after repeated administration of CHS-Na.[5-7] In addition, Prosky and O'Dell[8] found trace amounts of dicyclohexylamine (DCHA) in the urine of rats receiving long-term administration of cyclamate at low levels.

We were interested in the findings[9-11] that DCHA possesses carcinogenic activity. The purpose of the present study was to identify DCHA, a metabolite of CHS-Na, in the urine after repeated oral administration of CHS-Na to rabbits and rats and to examine the metabolic fate of DCHA in both animals.

Experimental

Materials——Pure CHS-Na was obtained by repeated recrystallization of reagent-grade material, and dried *in vacuo* at room temperature for 6 h. *n*-Butyl benzoate was purified by distillation of a commercial product of reagent grade. Other reagents were of special grade.

Administrations of CHS-Na and DCHA and Collections of Urine and Feces——Male rabbits weighing about 2.5 kg and male Wistar rats weighing 200—250 g were used. CHS-Na dissolved in water (25 mg/ml) was administered orally by means of a stomach tube at a dose of 1 g/animal/d to rabbits for 10—20 d or at 100 mg/animal/d to rats for 23—42 d. The animals were housed in individual metabolic cages with diet[12] and water *ad libitum*, and the urine was collected in flasks.

DCHA dissolved in McIlvaine buffer at pH 6.0 (5 mg/ml or 2 mg/ml) was administered orally at a dose of 50 mg/animal to rabbits or 5 mg/animal to rats, and then the urine and feces were collected at 24 and 48 h after administration. DCHA dissolved in McIlvaine buffer at pH 6.0 (100 mg/ml or 2 mg/ml) was also injected intravenously at a dose of 10 mg/animal to rabbits or 1 mg/animal to rats, and the urine and feces were collected as described above.

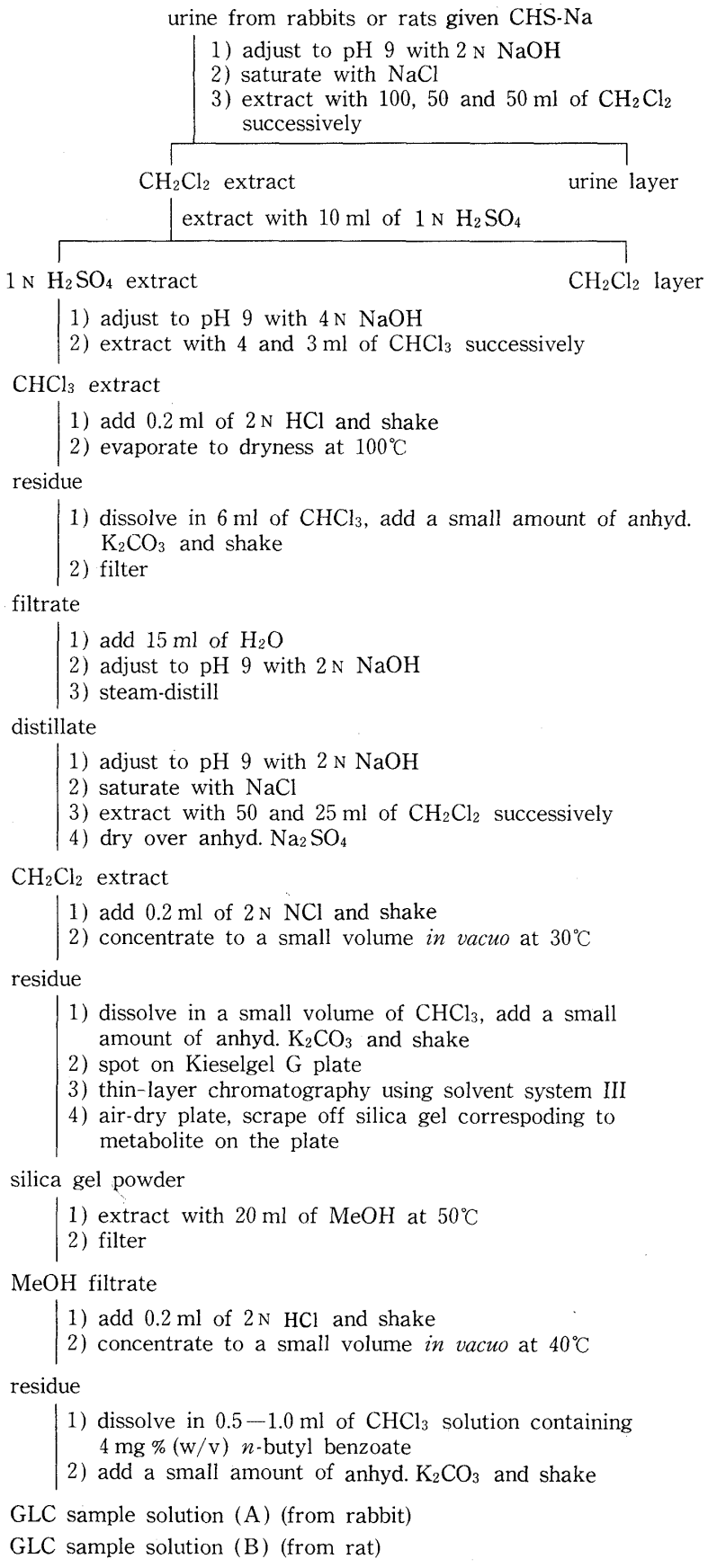

Fig. 1. Isolation Procedure for DCHA, a Metabolite of CHS-Na, from the Urine of Rabbit and Rat

***In Situ* Small Intestinal Absorption Procedure**——The *in situ* intestinal absorption experiment in rabbits was carried out in the same way as described in the previous paper[13] except for the length (about 40 cm) of the small intestine used and the sample solution, which was prepared by dissolving DCHA (30 mg/kg) in 150 ml of isotonic phosphate buffer (pH 7.4).

The procedure for studying the absorption of DCHA from *in situ* rat small intestine followed the method of Doluisio *et al.*[14] except for the use of 10 ml of sample solution (0.5 mg DCHA/ml McIlvaine buffer, pH 6.0).

***In Vitro* Experiment with Liver Homogenate Supernatant**——1) Rabbit: After being fasted overnight with access to water *ad libitum*, a rabbit was killed by exsanguination and the liver was removed. All subsequent manipulations were performed at 4°C. The liver was cut into small pieces and a 25% liver homogenate was prepared with a universal homogenizer in a KCl (1.15%)–phosphate buffer (0.01 M) at pH 7.4. After centrifugation for 30 min at $10000 \times g$, 4 ml of the supernatant fraction was added to an ice-cold mixture of 4 ml of buffer solution containing nicotinamide-adenine dinucleotide phosphate (NADP) (0.5 μM), glucose-6-phosphate (10.0 μM), nicotinamide (50.0 μM) and $MgCl_2$ (25 μM) and 1 ml of DCHA (1 mg/ml) in McIlvaine buffer (pH 6.0). The resulting mixture was incubated at 37°C for 3 h under aerobic (O_2) or anaerobic (N_2) conditions. At 1, 2, and 3 h, aliquots of the media were taken, and unchanged DCHA was determined.

2) Rat: Rats were killed by decapitation and the livers were removed immediately. The subsequent operation was identical to that described for rabbits except that 2 ml of the supernatant fraction was added to 2 ml of co-factor solution.

Thin-Layer Chromatographic Method——The usual ascending technique was employed with silica gel (DC-Alufolien Kieselgel 60, E. Merck AG.) plates, 10×20 or 20×20 cm in size, 0.25 mm in thickness, activated at 105°C for 1 h. The solvent systems employed were (I) *n*-butyl alcohol–acetic acid–water (4: 1: 2, v/v), (II) *n*-butyl alcohol–acetic acid–water (8: 1: 4, v/v), and (III) *n*-amyl alcohol–acetic acid–water (10: 2: 1, v/v). Spots were detected with ninhydrin reagent.

Apparatus and Conditions of Gas Liquid Chromatography (GLC)——A Shimadzu gas chromatograph (Model GC-3BF) equipped with a hydrogen flame ionization detector was used for analysis. The column was a $1.6\ m \times 3$ mm glass spiral tube containing 80—100 mesh Chromosorb W. H. P. coated with 20% PEG 20M and 2.5% KOH. The column temperature was maintained at 170°C. The flow rate of carrier gas (N_2) was 40 ml/min.

Analytical Procedure——DCHA excreted in the urine after repeated oral administration of CHS-Na was isolated according to the procedure shown in Fig. 1 and determined by the gas chromatographic method using *n*-butyl benzoate as an internal standard. The calibration curves were obtained by plotting the concentration of DCHA (10—50 μg and 100—500 μg) against the peak area ratio of DCHA to *n*-butyl benzoate. When DCHA (10—500 μg) was added to the control urine and the compound was analyzed by the method described above, the mean recovery of the compound was 80—90%.

Results and Discussion

Identification of DCHA in the Urine of Rabbits and Rats following Administration of CHS–Na

The sample solution (A), which was prepared by the method described in Fig. 1 from the urine of rabbits given repeated oral administration of CHS–Na, was analyzed by gas chromatography (GC) to examine the metabolites of CHS–Na. As shown in Fig. 2, one peak, which was not seen in control urine, was observed on the GC and the retention time showed good correspondence with that of authentic DCHA. In addition, the sample solution (A) was analyzed by TLC using three kinds of solvent systems. One spot, which was not found in the control urine, was detected on the TLC, and its *Rf* value was in fair agreement with that of authentic DCHA as shown in Table I.

Moreover, the sample solution (B), which was prepared from the urine of rats given repeated oral administration of CHS–Na, was analyzed by the GC and TLC. As shown in Fig. 2 and Table I, the GC and TLC showed the presence of DCHA in the sample solution (B).

Thus, these results showed that DCHA, a metabolite of CHS–Na, is present in the urine of rabbits and rats given CHS–Na repeatedly.

Quantitative Determination of DCHA in the Urine of Rabbits and Rats following Administration of CHS–Na

We determined DCHA in the urine of rabbits and rats receiving repeated oral administration of CHS–Na. Only a small amount of DCHA (mean 0.041×10^{-3} % of the dose) was excreted in the urine of rabbits given CHS–Na (1 g/d) for 10—20 d, as shown in Table II. In

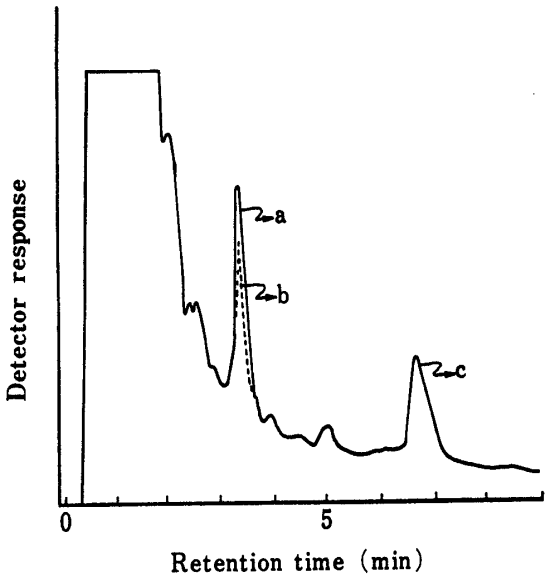

Fig. 2. Gas Chromatogram of DCHA from Sample Solutions (A) and (B)

Peak: a, DCHA from sample solutions (A) and (B);
b, authentic DCHA;
c, *n*-butyl benzoate (internal standard).

addition, the mean amount of DCHA excreted in the urine of rats given CHS–Na (100mg/d) for 23—42 d was 0.143×10^{-3} % of the dose, as shown in Table II. From these results, the urinary excretion of DCHA in rats was about 3.5 times that in rabbits, although the urinary excretion of DCHA was very small in both species. This is consistent with the report of Prosky and O'Dell[8] that DCHA accounted for less than 0.1% of the radioactivity excreted in the urine after intubation of ^{14}C-cyclamate in rats.

Metabolic Fate of DCHA

To examine further the finding that the amount of DCHA excreted in the urine of rabbits and rats given CHS–Na orally was very small, we investigated the metabolic fate of DCHA in rabbits and rats. First, the *in situ* absorption of DCHA from the small intestines of rabbits and rats was examined. As shown in Fig. 3, the absorption rate constants of DCHA

TABLE I. Thin-Layer Chromatography of DCHA in the Urine after Oral Administration of CHS-Na to Rabbits and Rats

Compound	*Rf* Solvent system[a]		
	I	II	III
Metabolite from sample solution (A)[b]	0.74	0.68	0.59
Metabolite from sample solution (B)[c]	0.74	0.70	0.60
Authentic DCHA	0.74	0.69	0.61

a) Solvent system I, *n*-butyl alcohol-acetic acid-water (4:1:2, v/v); II, *n*-butyl alcohol-acetic acid-water (8:1:4, v/v); III, *n*-amyl alcohol-acetic acid-water (10:2:1, v/v).
b) Prepared from the urine of rabbits given CHS-Na.
c) Prepared from the urine of rats given CHS-Na.

from the small intestines of rabbits and rats were 0.44 and 0.33 h^{-1}, respectively. These data show that the intestinal absorption of DCHA is quite rapid in both species.

Unchanged DCHA (% of the dose) excreted in the urine and feces of rabbits during 2 d after administration of DCHA amounted to 0.08 and 0.30%, respectively, for oral administration of DCHA (50 mg/animal), and 4.55 and 0.47%, respectively, for intravenous administration of DCHA (10 mg/animal), as shown in Table III. In addition, unchanged DCHA (% of the dose) excreted in the urine and feces of rats during 2 d amounted to 5.88 and 0.25%, respectively, for oral administration of DCHA (5 mg/animal) and 14.84 and 0.59%, respectively, for intravenous administration of DCHA (1 mg/animal), as shown in Table IV. The appearance of unchanged DCHA in the feces after intravenous administration of DCHA to rabbits and rats suggested that a part of the administered DCHA was excreted in the bile. The urinary

TABLE II. Urinary Excretion of DCHA after Continuous Oral Administration of CHS-Na in Rabbits and Rats

Animal	No. of animals	Duration of admin. (d)	Total dose of CHS-Na (g)	Excreted DCHA (μg)	% excreted×10³ [a]
Rabbit [b]	2	10	20.0	8.3	0.046
	2	20	40.0	13.9	0.039
	1	14	14.0	4.6	0.037
				Mean±S.D.	0.041±0.004
Rat [c]	20	30	60.0	54.8	0.102
	13	23	29.9	35.6	0.132
	11	42	46.2	42.0	0.101
	13	24	31.2	44.1	0.157
	10	31	31.0	49.2	0.176
	13	28	36.4	62.7	0.191
				Mean±S.D.	0.143±0.035

a) The percent excretion is given in terms of CHS-Na equivalent.
b) The dose of CHS-Na was 1 g/animal/d.
c) The dose of CHS-Na was 100 mg/animal/d.

and fecal excretions of unchanged DCHA were relatively small in both animals, although the amounts of unchanged DCHA excreted were larger in rats than in rabbits. From the above results, it is suggested that DCHA may be relatively easily metabolized in raibbts and rats.

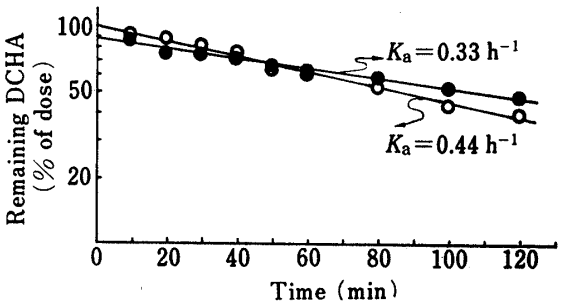

Fig. 3. Semilogarithmic Plots of Disappearance of DCHA from Small Intestine of Rabbit or Rat *in Situ*

Each value represents the mean for 2 animals.
—○—; rabbit.
—●—; rat.

An *in vitro* metabolism study of DCHA was performed using the liver 10000×*g* supernatant of rabbits and rats. As shown in Fig. 4(a), DCHA was rapidly metabolized under aerobic conditions in rabbit liver supernatant, but was little metabolized under anaerobic conditions. As shown in Fig. 4(b), the metabolism of DCHA in rat liver supernatant was slower than that in rabbit liver supernatant. From the *in vitro* metabolism and intravenous administration experiments, it is considered that DCHA is primarily metabolized by oxidative reaction in the liver of rabbits and rats. The finding that

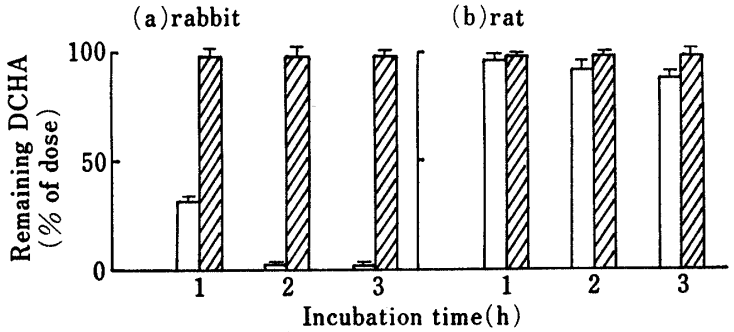

Fig. 4. Metabolism of DCHA in Hepatic 10000×*g* Supernatant Fraction of Rabbit and Rat

Each value is the mean±standard deviation for 2 or 3 experiments.
□; under aerobic conditions.
▨; under anaerobic conditions.

TABLE III. Urinary and Fecal Excretions of Unchanged DCHA after a Single Oral or Intravenous Administration of DCHA in Rabbits

	Day after admin.	Excreted DCHA (μg)[a]		% excreted[a]	
		Oral[b]	Intravenous[c]	Oral[b]	Intravenous[c]
Urine	1	24±12	444±112	0.05±0.03	4.44±1.13
	2	13±8	10±4	0.03±0.02	0.11±0.05
	Total	37±12	454±112	0.08±0.03	4.55±1.12
Feces	1	134±54	45±21	0.27±0.11	0.45±0.21
	2	12±3	2.49±1.76	0.03±0.01	0.02±0.02
	Total	146±57	47±21	0.30±0.12	0.47±0.21

a) Each value represents the mean ± standard deviation for 3 to 5 animals.
b) The dose of DCHA was 50 mg/animal.
c) The dose of DCHA was 10 mg/animal.

TABLE IV. Urinary and Fecal Excretions of Unchanged DCHA after a Single Oral or Intravenous Administration of DCHA in Rats

	Day after admin.	Excreted DCHA (μg)[a]		% excreted[a]	
		Oral[b]	Intravenous[c]	Oral[b]	Intravenous[c]
Urine	1	289±65	142±16	5.79±1.30	14.20±1.61
	2	4.38±0.88	6.38±0.63	0.09±0.02	0.64±0.07
	Total	294±66	148±17	5.88±1.32	14.84±1.65
Feces	1	7.01±0.33	4.23±0.97	0.14±0.01	0.42±0.10
	2	5.16±0.34	1.67±1.18	0.11±0.01	0.17±0.12
	Total	12.17±0.46	5.90±1.72	0.25±0.01	0.59±0.17

a) Each value represents the mean ± standard deviation for 4 or 5 animals.
b) The dose of DCHA was 5 mg/animal.
c) The dose of DCHA was 1 mg/animal.

the urinary excretion of unchanged DCHA after oral or intravenous administration of DCHA is less in rabbits than in rats may be explained by the difference in the metabolic rates of DCHA in the two species.

Thus, the finding that only a small amount of DCHA is excreted in the urine after repeated oral administration of CHS–Na to rabbits and rats may be due to further metabolism of the produced DCHA.

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References and Notes

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