Notes

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Studies on Synthetic Sweetening Agents. XIV.[1] Metabolism of Sodium Cyclamate. (3). On Metabolites of Sodium Cyclamate in Human

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Some workers[3–5] have already reported on the metabolism of sodium cyclamate (CHS–Na), and those reports showed that CHS–Na was excreted largely unchanged in both laboratory animals and human receiving the drug orally, intravenously, or intraperitoneally. However, the problem on the metabolism of CHS–Na has attracted attention of many workers since recent reports have shown that a cyclohexylamine can be a metabolite of CHS–Na in humans.[6–7] Leahy, *et al.*[7] reported that four of the 35 subjects, who had been given a single dose of 1 g CHS–Na, excreted cyclohexylamine in amounts corresponding to 0.2—3.1% of the dose of CHS–Na. In the previous paper[6] of this series, the authors reported the recovery in the urine of a human volunteer of an amount of cyclohexylamine which was equivalent to approximately 0.7% of an orally administered dose of CHS–Na. Furthermore, the previous study[1] demonstrated that cyclohexylamine, cyclohexanol, and cyclohexanone were excreted in the urine of rabbit and rat which had received CHS–Na orally.

The present report deals with a study on some metabolites of CHS–Na in human.

Experimental

Materials——CHS–Na was obtained by repeated recrystallization of pure reagent grade one, and dried at 105° for 2 hr. Cyclohexylamine, cyclohexanol, cyclohexanone, isoamylacetate, and *n*-butylether were purified by distillation of commercial products of reagent grade, bp 133—134°, bp 158°, bp 155°, bp 142°, and bp 141° respectively. Cyclohexyl 2,4-dinitrophenylhydrazone was prepared from cyclohexanone and 2,4-dinitrophenylhydrazine according to the method of Allen,[8] mp 158—159°. Cyclohexyl 3,5-dinitrobenzoate was prepared from cyclohexanol and 3,5-dinitrobenzoyl chloride according to the method of Siebenmann, *et al.*,[9] mp 111—113°. β-Glucuronidase was purchased from Nutritional Biochemicals Corporation, 70000—100000 units per g.

Administration of Drug and Collection of Urine——A single dose of 2 g CHS–Na was administered orally to each human volunteer, and the urine was collected in the flask containing toluene for preventing putrefaction for 24 hr.

Paper Chromatographic Method——The usual ascending technique was employed with use of Toyo Roshi No. 50. Solvent systems employed were (I) decalin–dimethylformamide, (II) *n*-heptane–MeOH, (III) *n*-BuOH saturated with 1N AcOH, and (IV) *n*-PrOH–28% NH_4OH (7:3). The chromatographies using solvent systems of (I) and (II) were carried out according to the methods of Sundt, *et al.*[10] and Meigh,[11]

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human urine (about 500 ml)

1) basified to pH 13 with 2N NaOH
2) saturated with NaCl
3) extracted with 200 and 100 ml of methylene chloride successively

methylene chloride extract

extracted with 10 ml of 0.5N HCl saturated with NaCl

0.5N HCl extract	methylene chloride layer
1) basified to pH 13 with 2N NaOH 2) extracted with 4 and 2 ml of $CHCl_3$ successively	1) dried over anhyd. Na_2SO_4 2) evaporated to dryness at 55°
$CHCl_3$ extract	residue
1) added 0.2 ml of 2N HCl, shaken 2) evaporated to dryness	dissolved in 0.1—0.5 ml of 1%(w/v) *n*-butylether $CHCl_3$ solution
residue	Sample solution (Bs, B, C, D)

1) dissolved in 0.5—5.0 ml of 30 mg%(w/v)isoamylacetate $CHCl_3$ solution
2) added a small amount of anhyd.K_2CO_3, shaken

sample solution (As, A)

Fig. 1. Preparation Procedures of Sample Solution (As), (Bs), (A), (B), (C) and (D)

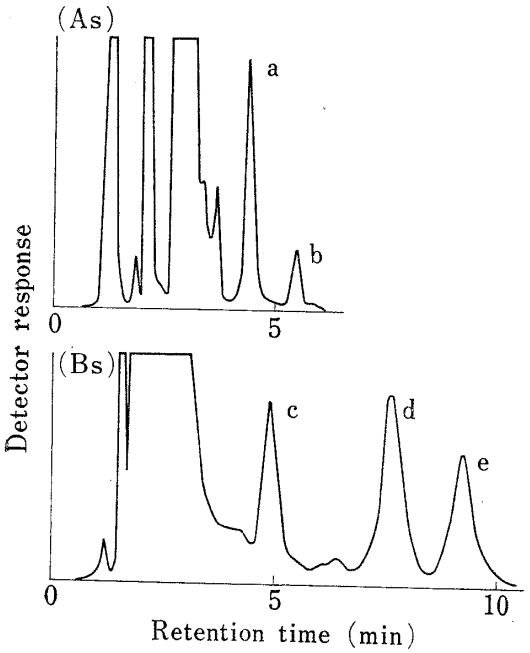

Fig. 2. Gas Chromatogram of Sample Solution (As) and (Bs)

apparatus: Shimadzu Model GC-3AF
peak: a) isoamylacetate (internal standard)
b) cyclohexylamine
c) *n*-butylether (internal standard)
d) cyclohexanone
e) cyclohexanol
condition: (As) temp.—column 130°
gas flow rate—N_2 30 ml/min, H_2 30 ml/min, air 1000 ml/min
sens.—100, sample size—2 μl
(Bs) temp.—column 140°
gas flow rate—N_2 30 ml/min, H_2 22 ml/min, air 1000 ml/min
sens.—100, sample size—2 μl

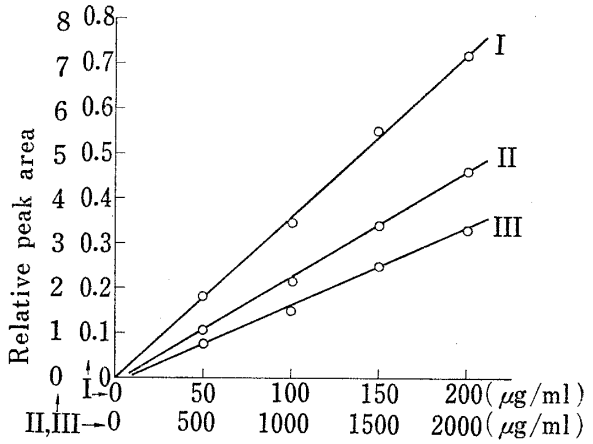

Fig. 3. Calibration Curves of Cyclohexylamine, Cyclohexanol, and Cyclohexanone

I : cyclohexylamine
II : cyclohexanone
III : cyclohexanol

respectively. Cyclohexyl 3,5-dinitrobenzoate was detected in ultraviolet light as dark spot on a yellow fluorescent background after spraying a methanolic solution of Rhodamine B, 20 mg per liter. Cyclohexylglucuronide was detected as deep-blue spot by spraying a paper with a fresh solution of naphthoresorcinol (2% in 33% aqueous trichloroacetic acid), drying at room temperature and then heating at 100—105° for about 30 min.

Thin-Layer Chromatographic Method——The usual ascending technique was employed with use of silicagel (Kieselgel G, E. Merck AG.) plates, 10×20 cm in size, 0.25 mm in thickness, activated at 105° for 1 hr. The solvent systems employed were (V) benzene–ligroin (1:1), (VI) benzene–ligroin (2:1), and (VII) benzene–petroleum ether (1:1).

Apparatus of Gas-Liquid Chromatography——A Shimadzu Model GC-1B dual column gas chromatograph equipped with a hydrogen flame ionization detector and a Shimadzu Model GC-3AF gas chromatograph were used in this studies. The carrier gas was nitrogen. The columns were 300 cm×4 mm *i.d.* stainless steel U-tube (for GC-1B) and 300 cm×3 mm *i.d.* stainless steel coil-tube (for GC-3AF) containing a packing of 20% PEG 20M and 2.5% NaOH on 60—80 mesh Shimalite.

The Model GC-1B was used for the identification of some metabolites of CHS-Na, and the Model GC-3AF for the quantitative investigation of cyclohexylamine, cyclohexanol, cyclohexanone, and conjugated cyclohexanol as the metabolites of CHS-Na.

Determination Methods of Cyclohexylamine, Cyclohexanol, and Cyclohexanone in the Urine——Each synthetic sample was prepared by accurately adding 50—200 μg of pure cyclohexylamine, cyclohexanol, and cyclohexanone respectively to about 500 ml of human blank urine. The sample solutions (As and Bs) were prepared from the above synthetic sample according to the procedures described in Fig. 1. At the fixed sensitivity and range of the instrument, 2 μl of sample solution (As or Bs) was injected into the gas chromatography. Those typical gas chromatograms were shown in Fig. 2. The peak areas were determined by triangulation. The calibration curve of cyclohexylamine was obtained by plotting the concentration of cyclohexylamine against the peak area ratio of cyclohexylamine to isoamylacetate (internal standard), and the calibration curves of cyclohexanol and cyclohexanone were obtained by the same manner but *n*-butylether was used as internal standard.

The sample solutions (A and B) were prepared from the urine of human receiving CHS-Na according to the methods described in Fig. 1 and submitted to gas chromatography respectively. The sample solution (A) was used for the determination of cyclohexylamine, and the sample solution (B) for the determination of cyclohexanol and cyclohexanone. The amounts of those metabolites were calculated by using the calibration curves shown in Fig. 3.

Determination Method of Conjugated Cyclohexanol in the Urine——Half a volume of 10N HCl was added to the urine (about 300 ml) of human receiving CHS-Na and the mixture was refluxed for 3 hr. The cooled mixture was saturated with NaCl and extracted with 200 and 100 ml of methylene chloride successively. The combined methylene chloride extract was washed with saturated $NaHCO_3$ solution, dried over anhyd. Na_2SO_4, and evaporated to dryness at 55°. The residue was dissolved in 0.1 ml of 1% (w/v) *n*-butylether $CHCl_3$ solution and the resulting solution was submitted to the gas chromatography. The total amounts of free cyclohexanol and cyclohexanol produced by acid hydrolysis of its conjugates in the urine were determined by using the calibration curve shown in Fig. 3, and then the amount of conjugated cyclohexanol in the urine was calculated by the following equation.

$$\text{Amount of conjugated cyclohexanol} = (C_t - C_f) \times \frac{276}{100}$$

where C_t is the total amount of free and conjugated cyclohexanol and C_f is the amount of free cyclohexanol, that is before acid hydrolysis.

Results and Discussion

Identification of Cyclohexanol and Cyclohexanone in the Urine

The sample solution (C) was prepared from one human (male) urine, which was collected for four successive 24 hours periods following the first dose after oral administration of 2 g CHS-Na per day for four days, using the procedure described in Fig. 1. The sample solution was submitted to gas chromatography in order to examine the metabolites of CHS-Na. As shown in Fig. 4, the gas chromatogram of sample solution (C) showed two peaks which were not identical with those of blank urine and had the same retention times with those of standard cyclohexanone and cyclohexanol, respectively.

For the purpose of examining in detail cyclohexanol and cyclohexanone as the metabolites of CHS-Na, further investigations were carried out as follows. The sample solution (D), which was prepared from one part of sample solution (C) according to the procedure described in Fig. 5, was submitted to paper chromatography using solvent systems of I, II, and III. One spot, which was not found in the blank, was detected in every chromatograms. Its *Rf* values on the paper chromatograms corresponded with those of standard cyclohexyl 3,5-dinitrobenzoate as shown in Table I. Also, the sample solution (E), which was prepared from the other part of sample solution (C) using the method described in Fig. 5, was examined by thin-layer chromatography using solvent systems of V, VI, and VII. One yellow spot was detected on the

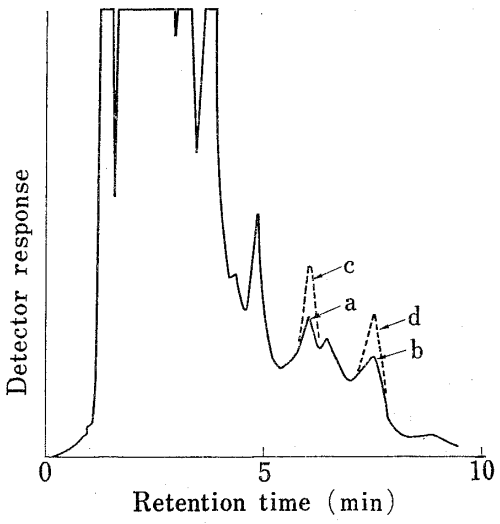

Fig. 4. Gas Chromatogram of Cyclohexanone and Cyclohexanol from Sample Solution (C)

apparatus: Shimadzu Model GC-1B
peak: a) one metabolite of CHS-Na
b) another metabolite of CHS-Na
c) standard cyclohexanone
d) standard cyclohexanol
condition:
temp.—column 150°, injector 230°, detector 200°
gas flow rate—N_2 65 ml/min, H_2 55 ml/min, air 1000 ml/min
sens.—1000, sample size—2 μl

thin-layer chromatograms, and its *Rf* values corresponded with those of standard cyclohexyl 2,4-dinitrophenylhydrazone as shown in Table II.

Identification of Cyclohexylglucuronide in the Urine

The sample solution (F) was prepared from one human (female) urine, which was collected for 24 hours after administering orally 2 g CHS-Na, using the method described in Fig. 6. The paper chromatography of the sample solution was investigated using solvent system of IV. The paper chromatogram showed one spot (*Rf* 0.61) which was not found in the blank and gave deep-blue by naphthoresorcinol reagent. Therefore, it was suggested that a glucuronide as a metabolite of CHS-Na was excreted in the urine of human receiving CHS-Na.

Then, 3 ml of sample solution (F) was spotted on the papers (40×40 cm) and chromatographed using solvent system of IV. The portion corresponding to *Rf* 0.61 was cut off, and each piece of the paper was eluted with warm water by the usual method. The eluate was concentrated to a small volume *in vacuo*. The resulting solution was incubated with about 10000 units of β-glucuronidase at

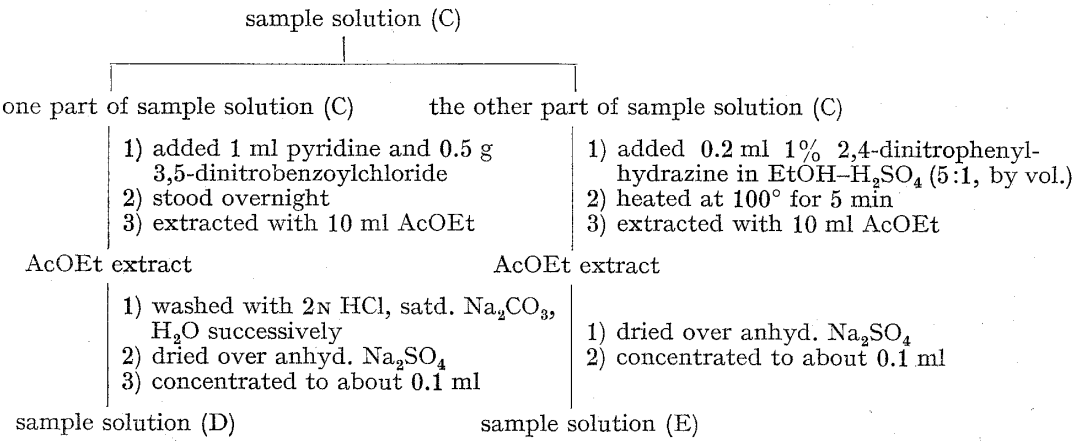

Fig. 5. Preparation Procedures of Sample Solution (D) and (E)

Table I. Paper Chromatography of Cyclohexanol as a Metabolite from Human Urine following Oral Administration of CHS-Na

Compound	Solvent system		
	I	II	III
		Rf	
Cyclohexyl 3,5-dinitrobenzoate	0.74	0.84	0.93
Metabolite of CHS-Na (as 3,5-dinitrobenzoate deriv.)	0.74	0.84	0.93

Table II. Thin-Layer Chromatography of Cyclohexanone as a Metabolite from Human Urine following Oral Administration of CHS-Na

Compound	Solvent system V	VI	VII
		Rf	
Cyclohexyl 2,4-dinitrophenylhydrazone	0.32	0.54	0.43
Metabolite of CHS-Na (as 2,4-dinitrophenylhydrazone deriv.)	0.32	0.54	0.43

38° for 3 hours, saturated with sodium chloride, and extracted with 10 ml of methylene chloride. The gas chromatogram of the methylene chloride solution, which was concentrated to a small volume, showed one peak corresponding with that of standard cyclohexanol as shown in Fig. 7. Furthermore, the cyclohexanol in the above methylene chloride solution was made up 3,5-dinitrobenzoate derivative according to the procedure described Fig. 5 and was submitted to paper chromatography using solvent systems of I and II. As shown in Table III, its *Rf* values corresponded with those of standard cyclohexyl 3,5-dinitro-

human urine
- 1) adjusted to pH 3—4 with AcOH
- 2) added satd.lead acetate
- 3) filtrated

filtrate
- 1) adjusted to pH 8 with 28% NH_4OH
- 2) added satd. basic lead acetate
- 3) filtrated

precipitate
- 1) suspended in 300 ml H_2O
- 2) treated with H_2S
- 3) filtrated

filtrate
- concentrated to a small volume *in vacuo* at 45°

residue
- 1) added 10 ml EtOH
- 2) filtrated

filtrate
- 1) evaporated to dryness
- 2) dissolved in 3 ml H_2O

sample solution (F)

Fig. 6. Preparation Procedure of Sample Solution (F)

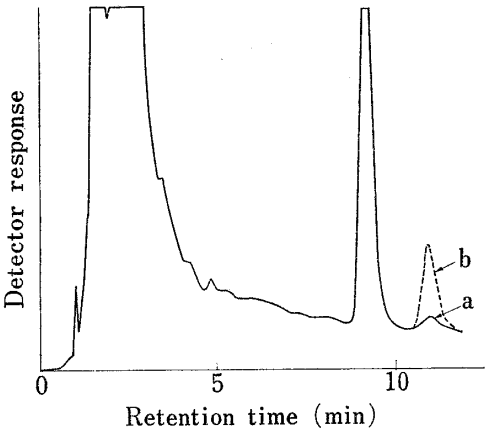

Fig. 7. Gas Chromatogram of Cyclohexanol obtained by Enzymatic Hydrolysis of Cyclohexylglucuronide

apparatus: Shimadzu Model GC-1B
peak: a) hydrolysate of CHS-Na metabolite
b) standard cyclohexanol
condition:
temp.—column 140°, injector 230°, detector 200°
gas flow rate—N_2 65 ml/min, H_2 55 ml/min, air 1000 ml/min
sens.—1000, sample size—2 μl

Table III. Paper Chromatography of Cyclohexanol obtained by Enzymatic Hydrolysis of Cyclohexylglucuronide in the Urine of Human receiving CHS-Na

Compound	Solvent system I	II
	Rf	
Cyclohexyl 3,5-dinitrobenzoate	0.74	0.84
Hydrolysate of CHS-Na metabolite (as 3,5-dinitrobenzoate deriv.)	0.74	0.83

benzoate. Thus it was found that cyclohexylglucuronide was excreted in the urine of human administered CHS–Na.

Quantitative Investigation of Cyclohexylamine, Cyclohexanol, Cyclohexanone, and Conjugated Cyclohexanol in the Urine

Five volunteers (3 males and 2 females) were given a single dose of 2 g CHS–Na orally. Each volunteer urine was collected for 24 hours after administering CHS–Na. The metabolites of CHS–Na in the urine, cyclohexylamine, cyclohexanol, cyclohexanone, and conjugated cyclohexanol, were determined according to the methods described in experimental.

As shown in Table IV, the results obtained indicated that cyclohexylamine, cyclohexanol, cyclohexanone, and conjugated cyclohexanol were found in the urines of all volunteers receiving CHS–Na, furthermore, that those metabolites excreted in the urine were a small amount.

Table IV. Urinary Excretion of the Metabolites in the 24 hr Urine of Human receiving Orally 2 g CHS-Na

Subject[a]	μg excreted			
	Cyclohexylamine	Cyclohexanol	Cyclohexanone	Conjugated cyclohexanol
K.I. (27—52) ♂	82	150	216	1710
K.S. (25—52) ♂	628	134	212	3990
S.K. (38—51) ♂	10800	45	82	2660
A.S. (21—47) ♀	2370	25	20	3080
Y.O. (21—51) ♀	40	20	10	5140

a) Bracketed quantities are subject's age in years followed by body weight in kilograms.

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Studies on the Glucaric Acid Pathway in the Metabolism of D-Glucuronic Acid in Mammals. V.[1,2] Stimulatory Effect of Diphenylhydantoin and Phenobarbital on the D-Glucaric Acid Synthesis in Man

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Recently an alternative pathway of D-glucuronic acid conversion, in addition to the conversion to L-ascorbic acid or to L-xylulose,[4] which involves the oxidation of D-glucuronolactone (I) to D-glucaric acid (III) by D-glucuronolactone dehydrogenase, has been demon-

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