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Conversion of Cyclamate to Cyclohexylamine in Guinea Pig[1]

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Continuous oral administration of sodium cyclamate(CHS-Na) to guinea pig caused the gradual increase of urinary excretion of cyclohexylamine(CHA) after a certain period. The amount of CHA excreted was not only different in individuals, but also dependent on each experiment. It was diminished when CHS-Na was administered orally with antibiotics or intraperitoneally. It was shown from the incubation experiments that apparent accumulation of CHA was observed by cecal contents or feces from the animal excreting CHA in urine by oral administration of CHS-Na, but not from that diminished the excretion by the above conditions. Any *in vitro* study using animal tissues failed to detect the accumulation of CHA. Consequently it may be probable to assume the participation of duct microflora in the conversion of CHS-Na to CHA in guinea pig.

The urinary excretion of cyclohexylamine (CHA) in laboratory animals[3] and humans[4] after oral administration of sodium cyclamate (CHS-Na) has already been established by many workers, since the original report[5] by Kojima and Ichibagase in 1966. Besides CHA, cyclohexanol (CHnol) and cyclohexanone (CHnone) were excreted in urine of rabbit and rat[6] and furthermore cyclohexylglucuronide in human urine.[7]

In the previous paper[8] the present authors ascertained that the variable amounts of CHA were excreted in urine of human volunteers ingested CHS-Na or taking usual meals. Now it is very interesting to know the site and mechanism of the biotransformation of CHS-Na to CHA, but it has not yet been well elucidated whether this conversion occurred in animal tissues or duct microflora, except a few brief reports supporting the respective standpoints.[6,9]

In this paper the urinary excretion was determined in the guinea pig to which CHS-Na was orally administered with and without antibiotics, or intraperitoneally. At the same time the formation of CHA was compared in the *in vitro* study of feces, cecal contents and liver homogenate. As the results of these experiments it was suggested that duct microflora took an important part in the conversion of CHS-Na to CHA in guinea pig.

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Experimental

Analytical Methods——CHA, CHnol and CHnone were determined by gas chromatography using Shimadzu-4APF with dual flame ionization detectors, 3 mm×200 cm stainless steel column packed with 10% Carbowax 20M plus 2.5% NaOH on 60—80 mesh Chromosorb G, column temperature 90°, injector temperature 130°, detector temperature 145°, carrier gas(nitrogen) flow rate 50 ml/min and injected sample 4 μl. The retention times of CHA and *n*-tridecane, the inner standard, were 5.1 min and 9.1 min respectively. Those of *n*-dodecane, the inner standard, CHnone and CHnol were 5.1 min, 10.7 min and 16.2 min respectively.

Materials——Sodium cyclamate(Yoshitomi Pharmaceutical Co.). Other reagents were commercials (reagent grade), if necessary, redistilled before use.

Preparation of Sample——CHA fraction was prepared from urine in the same procedure previously described(Method-2)[8] and analysed by gas chromatography. CHnol and CHnone fractions were prepared by concentrating the first $CHCl_3$ layer in Method-2 to approximate 0.2 ml *in vacuo* at less than 30° and determined by gas chromatography after adding 0.05 ml of the inner standard solution containing 25 μg of *n*-dodecane in $CHCl_3$. Usually 24 hr urine was treated after dilution to 60 ml with water. The standard curves of CHnone and CHnol recovered from urine were shown in Fig. 1.

***In Vitro* Study of Feces and Cecal Contents**——Feces and cecal contents of guinea pig were homogenized with twice amounts of 1% CHS-Na and 20—30 ml of the homogenate in a test tube (ϕ18×200 mm) with a cotton plug was incubated under the semi-anaerobic condition for several days at 30°. After dilution of the incubated homogenate to 60 ml with water, CHA accumulated was determined by the same procedure as in the case of urine.

***In Vitro* Study of Tissues**——Liver, kidney and small intestine of guinea pig to which CHS-Na was orally administered for a long time were homogenized with twice amounts of 0.1M phosphate buffer (pH 7.0). Twenty ml of the homogenate containing 0.1 g of CHS-Na was incubated in a 100 ml of Erlenmeyer flask for several hours at 37°. After dilution of the incubated homogenate to 60 ml with water, CHA formed was determined by the method described above.

In order to prolong the incubation time, liver was aseptically removed from the body, homogenized with twice amounts of sterilized Krebs–Ringer phosphate buffer(pH 7.0) containing 1% CHS-Na and incubated for 3 days at 30°. As all the procedure was aseptically done with caution, the putrefaction could be avoided during the incubation period.

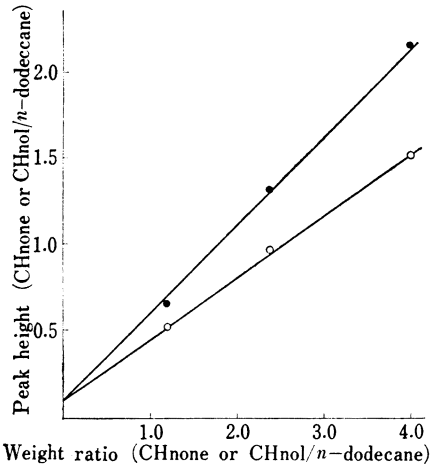

Fig. 1. Standard Curves of CHnone and CHnol recovered from Urine
—●—: CHnone; —○—: CHnol

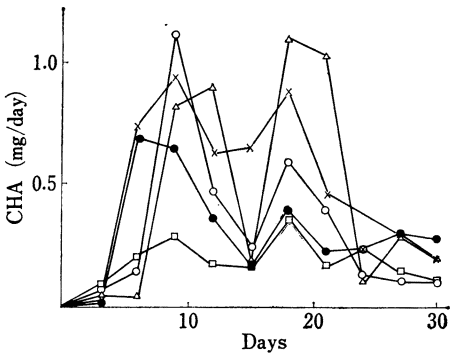

Fig. 2. Excretion of CHA in Urine of Guinea Pigs taking 1% CHS-Na as Drinking Water
—○—: No. 1; —×—: No. 2; —●—: No. 3; —△—: No. 4; —□—: No. 5

Result

Urinary Excretions of CHA, CHnol and CHnone

Five male guinea pigs (250—300 g) were given 1% CHS-Na as drinking water and ground commercial feed *ad libitum*. They actually took about 20—30 ml of 1% CHS-Na every day. The animals were placed in individual metabolism cages which permitted separate collections of urine from feces. Determining CHA excreted in 24 hr urine every second

day, as shown in Fig. 2, the amount began to increase rapidly after several days, followed the variable excretion of CHA. Such a type of excretion pattern was thought to be rather a general phenomenon, because the same results could be always obtained in other experiments which would be described later.

In another experiment we found the unexpected result which six of ten male guinea pigs tested died between two weeks and one month after the initiation of oral administration of CHS-Na, with rapid increase of urinary excretion of CHA and decrease of body weight in several days before the death. Some animal excreted more than 70 mg of CHA per day just before the death (Fig. 3).

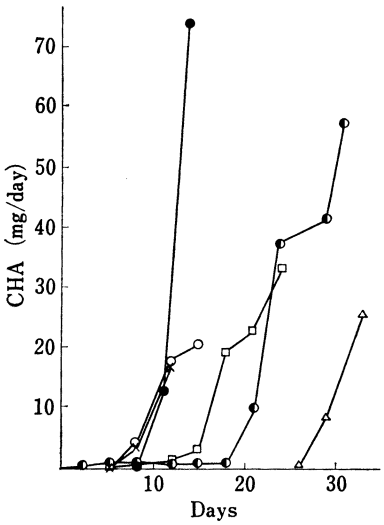

Fig. 3. Excretion of CHA in Urine of Six died Guinea Pigs taking 1% CHS-Na as Drinking Water

—○—: No. 1 (15); —×—: No. 3 (12); —●—: No. 6 (13); —△—: No. 8 (33); —□—: No. 9 (24); —◑—: No. 10 (31)
The values in parentheses are the days of death.

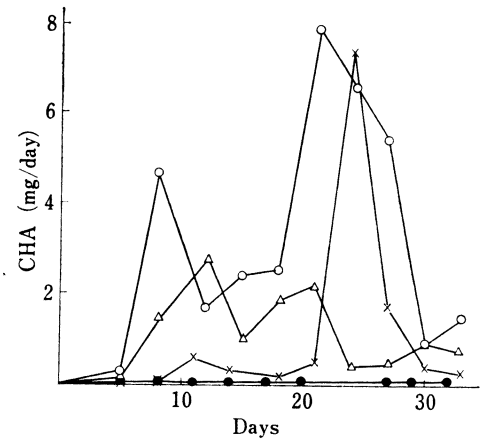

Fig. 4. Excretion of CHA in Urine of Four survived Guinea Pigs taking 1% CHS-Na as Drinking Water

—○—: No. 2; —×—: No. 4; —●—: No. 5; —△—: No. 7

In this experiment, as shown in Fig. 4, even three survived animals except one excreted larger amount of CHA than the previous experimental group shown in Fig. 2.

The urinary excretion of CHnol (0.02—0.5 mg/day) had also a tendency to increase before the death, but there were some exceptions (Fig. 5 and 6). Little or no CHnone was excreted, if any, less than 0.09 mg/day.

As such a extreme phenomenon which guinea pigs died accompaning large amount of urinary excretion of CHA could not be obtained thereafter, it might be probable to assume that some bacteria capable to metabolize CHS-Na to CHA anomalously grew in the guts and most of the animals were infected by them, taking into consideration of the other experimental results which would be described later.

Comparison of Urinary Excretion of CHA in Intraperitoneal or Oral Administration of CHS-Na with and without Antibiotics

Seven male guinea pigs (250—300 g) were placed in individual metabolism cages. 1% CHS-Na was given as drinking water in group A (No. 1, 2 and 3), 1% CHS-Na containing 0.01% streptomycin sulfate and 0.01% fragiomycin was given as drinking water in group B (No. 4 and 5) and 2 ml of 10% CHS-Na was intraperitoneally injected every day in group C

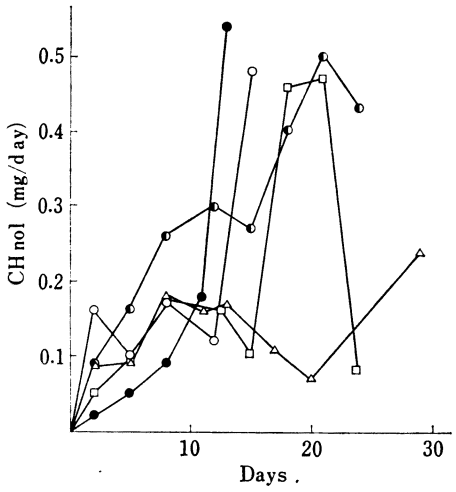

Fig. 5. Excretion of CHnol in Urine of Six died Guinea Pigs taking 1% CHS-Na

—○—: No. 1 (15); —×—: No. 3 (12);
—●—: No. 6 (13); —△—: No. 8 (33);
—□—: No. 9 (24); —◑—: No. 10 (31)
The values in parentheses are the days of death.

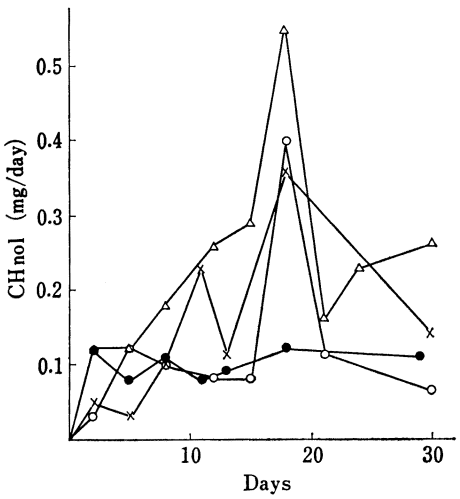

Fig. 6. Excretion of CHnol in Urine of Four survived Guinea Pigs taking 1% CHS-Na as Drinking Water

—○—: No. 2; —×—: No. 4;
—●—: No. 5; —△—: No. 7

(No. 6 and 7). All the animals were permitted to give drinking water and ground commercial feed *ad libitum*. In these conditions they took about 20—30 ml of the drinking water every day. Twenty four hours urine was collected every second day and CHA excreted was determined.

As Figure 7 shows, the guinea pigs in group A began to excrete CHA in urine after about 10 days followed the repetition of increase and decrease. The maximum values were 1.24 mg/day (39th day) in No. 1, 2.60 mg/day (36th day) in No. 2 and 3.85 mg/day (33rd day) in No. 3 respectively. On the contrary the excretions of CHA in group B and C were less than 0.1 mg/day.

The weights of guinea pigs in group B did not increase enough because of the long term administration of antibiotics.

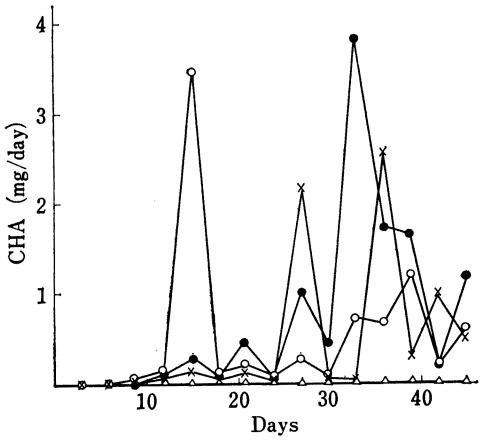

Fig. 7. Excretion of CHA in Urine of Guinea Pigs taking 1% CHS-Na as Drinking Water with and without Antibiotics or intraperitoneally injected 0.2 g CHS-Na every Day

group A (No. 1, 2, 3) 1% CHS-Na (*per os*); group B (No. 4, 5) 1% CHS-Na+antibiotics[a] (*per os*); group C (No. 6, 7) 0.2 g CHS-Na (*i.p.*); —○—: No. 1; —×—: No. 2; —●—: No. 3; —△—: No. 4, 5, 6, 7
a) 0.01% streptomycin sulfate+0.01% fragiomycin

In Vitro Study of Cecal Contents

All the cecal contents were removed from cecum of both guinea pigs given 1% CHS-Na and water (control), and homogenized with 150 ml of 1% CHS-Na. Thirty ml of homogenate in an Erlenmeyer flask with a cotton plug was shaken on a rotary shaker at 30° (aerobic condition). Another 30 ml of homogenate in a test tube (ϕ 18×200 mm) with a cotton plug was kept at 30° (semi-anaerobic condition). CHA accumulated in the homogenate was determined at t=0 and 4 days. A large amount of CHA was accumulated in the homo-

Table I. Accumulation of CHA in the Incubation of Cecal Contents with CHS-Na under Aerobic and Semi-anaerobic Conditions

Incubation time (days)	CHA accumulated (μg/30 ml)			
	CHS-Na dose		Control	
	Aerobic	Semi-anaerobic	Aerobic	Semi-anaerobic
0	1	1	4	4
4	572	3100	6	6

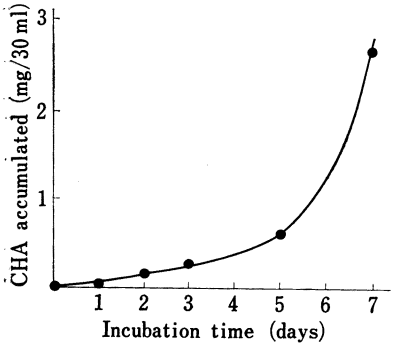

Fig. 8. Accumulation Curve of CHA in the Incubation of Cecal Contents with CHS-Na under Semi-anaerobic Condition

genate of CHS-Na dosed animal, especially under the semi-anaerobic condition (Table I) and the CHA accumulation rapidly increased after several days (Fig. 8).

From these results, all the following *in vitro* studies of cecal contents and feces were incubated under semi-anaerobic condition for 5 or 6 days.

Comparing the *in vitro* study of group A, B, C and control, as shown in Table II, a large amount of CHA was accumulated in group A, while little amount in group B, C and control.

The cecal contents of accidentally died animal in group A (No. 2 in Table II) showed the high level accumulation of CHA at $t=6$ days and even $t=0$. It was suggested from this fact that the absorption of CHA from duct had ceased after the death of animal

Table II. Comparison of CHA accumulated in the *in Vitro* Study of Cecal Contents of Group A, B, C and Control Guinea Pigs

Guinea pig used		CHA accumulated (μg/30 ml)		
Group	No.	$t=0$ (day)	$t=3$ (days)	$t=6$ (days)
A	2[a)]	1720	9400	35400
A	3	11.2	411	643
B	5[a)]	4.8	—	0
C	7	5.4	—	25.8
Control	—	0	19.2	0

a) accidentally died

causing the accumulation of CHA in the cecum and that the anomalous growth of duct bacteria capable to utilize CHS-Na furthered a striking increase of CHA accumulation during the incubation time. In the case of group B (No. 5 in Table II) little or no accumulation was shown at $t=0$ or 6 days by the effect of antibiotics.

In Vitro Study of Feces

The same experiment of *in vitro* study was performed by using feces of guinea pig under the same conditions as the cecal contents. In the case of group A a significant amount of CHA was accumulated during the incubation, but in group B and C only 1/10—1/25 of that as shown in Table III.

In Vitro Study of Tissue

In order to ascertain whether CHA is formed in tissue, CHS-Na was incubated with the homogenates of liver, kidney and small intestine of guinea pig which CHA was excreted

Table III. Comparison of CHA accumulated in the *in Vitro* Study of Fecal Homogenates of Group A, B and C Guinea Pigs

Exp.	Guinea pig used		CHA accumulated (μg/30 ml)		CHA excreted in urine (μg/day)
	group	No.	$t=0$ (day)	$t=5$[a] (days)	
I	A	1	24.0	430	269
	B	5	0	7.2	5.6
	C	7	0	31.4	7.6
II	A	2	132	1800	829
	B	4	4.8	16.4	5.8
	C	6	4.8	96.6	94.2

a) $t=6$ (days) in Exp. II

in the urine, but no CHA was accumulated at least for 5 hr. No positive results could be obtained in the incubation experiment using microsome or lysozyme fraction of liver. Furthermore the formation of CHA was not observed for the prolonged incubation time (3 days) in the experiment of liver homogenate under the aseptic condition described in the Method (Table IV).

Table IV. Comparison of CHA accumulated in the *in Vitro* Study of Liver Homogenate and Cecal Contents

Guinea pig used		Homogenate	CHA accumulated (μg/20 ml)[a]	
Group	No.		$t=0$ (day)	$t=3$ (days)
A	3	liver	11.4	8.4
		cecal contents	11.2	411
Control	—	liver	7.2	8.2
		cecal contents	0	19.2

a) (μg/30 ml) in cecal content

Discussion

When CHS-Na was orally administered to guinea pig every day, the urinary excretion of CHA was gradually increased as observed in other animals and humans. The amount of CHA excreted was not only different in individuals, but dependent on each experiment; some time the range was less than one mg, other time several mg and in one anomalous case reached several tens mg. Such a difference of CHA excretion level in each experiment might be explained by a possibility that the animals breeded in the same place and time mutually had the similar pattern of duct microflora metabolizing CHS-Na to CHA.

Since Kojima and Ichibagase[5] demonstrated the excretion of CHA in urine of experimental animals which CHS-Na was orally administered, it has been a very interesting problem to elucidate the site and mechanism of biotransformation of CHS-Na to CHA. Some workers insisted the participation of tissue enzyme,[6] while others that of duct microflora.[9]

In this paper we ascertained that the guinea pigs which CHS-Na were administered intraperitoneally or orally with antibiotics diminished the excretion of CHA in urine. At the same time in the incubation experiments of feces and cecal contents with CHS-Na, remarkable amount of CHA was accumulated only in the case of the animal to which CHS-Na was orally administered and CHA was excreted in urine, but little or no CHA was accumulated in the case of simultaneous dose of antibiotics with CHS-Na or intraperitoneal dose of CHS-Na. On the contrary no CHA was formed in the incubation experiment of liver homogenate from any treated guinea pig.

From the result in this paper, it may be probable to think the participation of duct microflora in the conversion of CHS-Na to CHA; certain microorganisms in the duct were adapted to utilize CHS-Na during the daily contact with CHS-Na for a certain period. As the simultaneous administration of antibiotics with CHS-Na should disturb the normal growth of duct microflora and the intraperitoneal administration of CHS-Na should avoid the direct passage through duct, the diminish of urinary excretion of CHA in both cases would be explained by the assumption that the conditions allowing to grow such microorganisms have not been established.

Furthermore several strains of bacteria capable to assimilate CHS-Na were separated from feces of guinea pig which CHA was excreted in urine, and the study of the bacterial enzyme system degrading CHS-Na is in progress.

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